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STUDIES IN THE PHYSIOLOGY OF THE FUNGI

XVI. Some Aspects of Nitrogen Metabolism in Fungi[1]

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Introduction and Review of Literature

Few extensive, really fundamental contributions to our knowledge of the nitrogen metabolism of plants are to be found. Even to-day practically nothing is known of the course of the assimilatory processes beyond the beginning and end points. It is evident that an insight into this fundamental life process will be more readily obtained by a study of fungi rather than of the higher plants, because of the relatively greater ease of the application of pure culture methods to the former.

The nitrogen fixation of fungi may properly be considered first. A glance at the literature reveals many conflicting data. In spite of the great number of publications, covering a period of over 50 years, it was not until 1916 that a paper appeared which included a complete review of the literature and gave evidence of adequacy in the technique employed. The technique of Duggar and Davis ('16) was significant in that Kjeldahl flasks were used directly as the culture vessels, thus necessitating no transference of either the fungous mat or culture solution prior

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to the nitrogen determination, a precaution which was not observed in the work of previous investigators. As many determinations were made on control portions of the media as on cultures of the various fungi. Under the several conditions of the experiments the fixation of elementary N by *Aspergillus niger*, *Macrosporium commune*, *Penicillium digitatum*, *P. expansum*, and *Glomerella Gossypii* could not be demonstrated; whereas *Phoma Betae*, growing on mangel and sugar beet decoctions with sucrose, showed a definite fixation ranging approximately from 3 to 8 mgm. per culture. This latter fact lends weight to the data of Ternetz ('04) indicating the marked capacity of *Phoma radicis* for assimilating free nitrogen. It, moreover, "throws open the whole question for any and all fungi," and suggests the sphaeropsidaceous and mycorhizal forms as material for first investigation.

The other important contributions to the subject of nitrogen metabolism are here reviewed, the chronological order being observed in so far as it was found compatible with clearness. Critical evaluation of the cited articles is largely reserved for the discussion at the end of this paper. The work with bacteria is not considered. The recent developments of physiological technique, such as the determination of active acidity and the improved methods for determining sugars and the various forms of nitrogen, throw doubt on the validity of the interpretations of many of the results of earlier investigators whose criterion for the assimilability and nutritive value of a given compound was generally based solely on the amount of growth. One now avoids arbitrary cataloging of fungi as "ammonia organisms," "peptone organisms," "nitrate forms" and so on, as was done in the older texts on plant physiology.

That heterotrophic plants were early known to be capable of utilizing inorganic nitrogen is evident from the challenge of Pasteur to Liebig to grow any amount of ferment on a purely synthetic medium which had ammonium N as the sole N source. Duclaux ('64), speculating on the synthesis of proteins in the course of fermentation, said that it was highly improbable that it could follow by direct condensation of ammonia and sugar, but rather that there must precede a breaking up of the sugar

molecule into its very reactive fragments which would unite with the ammonia to form amino acids from which the proteins would arise.

Czapek ('02), in his extensive cultural studies with *Aspergillus niger*, used cane sugar as a carbon source and an almost all-inclusive list of possible N sources. Judging from the substances used, the per cent of N utilized, the appearance of the culture, and the dry weights of the fungous crops obtained in the presence and absence of sugar, he concluded that proteins are most easily synthesized from the amino acids and from those substances which most nearly resemble the amino acids. For example, the high utility of acetamid as an N source is explained by the fact that its structure approaches that of an amino acid.

"Die Eiweissynthese wird demnach aus Aminosäuren (und Diaminosäuren) am leichtesten und ausgiebigsten bewerkstelligt, wenn man gleichzeitig eine gute Kohlenstoffquelle, z. B. eine Zuckerart, darreicht. Es vermag also der Schimmelpilz aus irgend einer beliebigen Aminosäure viel leichter alle übrigen, welche als Bausteine des Eiweissmoleküls in Betracht kommen, zu bilden, als erst aus anderen Stickstoffverbindungen Aminosäuren synthetisch aufzubauen. Man kann ferner annehmen, da die Aminosäuresynthese als Vorstufe zur Eiweissbildung anzusehen ist, dass aus jenen Stoffen, welche am besten als N-Quellen dienen (hier besonders den Oxyfettsäuren), auch am leichtesten die Synthese der Aminosäuren bewerkstelligt werden kann—ein Gedanke, welcher in ferneren Untersuchungsreihe noch weiter verfolgt werden soll."

In a later experiment he used asparagin as the N source and varied the carbon source, employing in all 24 carbohydrates, higher alcohols, and organic acids. From the dry weights obtained, the extent of utilization of the asparagin, and the appearance of the culture, he supports the "Eiweissregeneration aus Asparagin und Kohlehydraten" hypothesis of Pfeffer ('72) and Borodin ('78), stating that protein synthesis is essentially the same in *Aspergillus niger* as in the seedlings of flowering plants. Loew also speculated on the significance of asparagin in nutrition, saying that a fungus fed this and sugar forms an aldehyde of aspartic acid which is then condensed to make the protein molecule.

The classic chemical work of Emil Fischer (K. Hoesch, '21), following closely upon that of Czapek, threw much light upon the constitution of the protein molecule and its manner of synthesis.

Lutz ('05), using a modified Raulin's solution as the basis of a culture medium, compared the assimilability by *Aspergillus niger* of NH_4 salts, amines, amides, and nitriles by obtaining dry weights of the felts for a given period of incubation. He concluded that amides were the most assimilable, exceeding the NH_4 salts; amines came next, and the order of their assimilability was in inverse ratio to the size of their molecule; while nitriles were of little value. He stated: "This conclusion is in perfect concordance with that which we know of the chemical constitution of these diverse bodies; those in which the molecule is the more simple theoretically ought to be and practically are the better source of N for the plants."

Ritter ('09) worked with 8 different fungi, and employed, as criteria, dry weight and the quantity of 0.1 *N* alkali required to neutralize 10 ml. of the culture fluid after growth of the organisms. He formulated the following conclusions: (1) The weaker and less poisonous the free acids the better NH_4 is taken up out of its mineral salts. (2) The development of fungi on NH_4-salt solutions is in direct proportion to their ability to withstand free acid. (3) In relation to the quantity of mineral acids they are capable of generating, the fungi are placed in 2 groups: (a) the mat-forming fungi, as *Aspergillus* and *Rhizopus*, which liberate more acid than would permit the germination of their spores, and (b) such fungi as various species of *Mucor*, which grow submerged and produce acid in concentration not inhibitive to spore germination. (4) *Aspergillus glaucus*, *Mucor racemosus*, and *Cladosporium herbarum*, designated "Nitratpilze," develop as well on ammonium (NH_4).N. (5) These 3 fungi, however, show a strongly evident capacity for NO_3 assimilation; *Aspergillus niger*, *Botrytis cinerea*, and *Penicillium* spp. are weaker in this capacity and produce greater growth on $(NH_4)_2SO_4$; a third group, represented by *Rhizopus nigricans*, *Mucor Mucedo*, and *Thamnidium elegans* refuse nitrates.

In the 1912 paper he continues his ('09) observations, here employing other carbon sources than the grape sugar of the former work. By an ingenious method of draining the culture fluid off the mat and flooding the fungus with an alkaline nitrate solution, then incubating 2 or 3 days, he demonstrated the significant re-

duction of nitrates to nitrites by the various fungi. The solution was tested qualitatively with Trommsdorf's reagent and metaphenylendiamin. Nitrite was considered an intermediate product in nitrate assimilation. Ammonia, however, evolving from further reduction could not be demonstrated. Because ammonia is a product of autolysis he warns against the error of interpreting the presence of this as an indication of further reduction, as was done by Schlösing and Müntz ('78), Hagem ('10), and others.

Ritter ('14) grew *Aspergillus niger* on a medium containing 10 per cent cane sugar as the carbon source, and NH_4NO_3 in concentrations of 0.4, 0.8, 1.6 per cent as the source of N. After incubation the mat was filtered off, washed, and dried, and on the filtrate and washings, made to volume, were determined NH_3 by distillation with MgO, RNO_3 by reduction with Zn and Fe, and acidity in terms of 0.1 *N* NaOH. The acidity was also calculated from the difference between the amounts of RNO_3 and NH_3 found in the culture. He found that the extent of the acidity attained in 3 to 4 days was such as to render further development of the fungus impossible, as shown by the dry weights. The quantity of acid produced in the media containing 0.8 and 1.6 per cent NH_4NO_3 approached 0.1 normality but in those having only 0.42 per cent it reached only 0.04 *N* in 2 days and fell to 0.002 *N* in 8 days, indicating, he thought, a utilization of HNO_3 by the fungus. Parallel experiments with the N sources, ammonium tartrate, and HNO_3 in the strength equivalent to 11.35 mg. of N per culture, showed that the free acid after 6 days of incubation at 32° C. was superior for *Aspergillus niger* and a *Penicillium*.

Hagem ('10) investigated many species of *Mucor* and divided them into 2 classes with respect to their ability to assimilate N from nitrates and nitrites. All forms that were capable of assimilating nitrates could also obtain their N from the NO_2 ion. Because of this and because all thrived on N supplied as ammonium salts, and because in all cultures ammonia accumulated in the culture medium, he assumed that in the process of nitrate assimilation nitrates are reduced to nitrites and further to ammonia. Species thriving on sucrose could not utilize this sugar

when amino acids were supplied as the sole N source. Amino acids, moreover, as the sole source of both C and N had very little value for these forms.

Kossowicz ('12), in his cultural studies with 10 fungi, including a *Botrytis*, several species of *Penicillium*, a *Phytophthora*, and 2 species of *Aspergillus*, found that KNO_2 formed a good N source in the presence of cane sugar or dextrose. All these forms likewise made good growth with urea or uric acid as the N source. The following exceptional things were noted. With cane sugar as the C source *Cladosporium herbarum* would grow neither on glycine nor hippuric acid, and *Penicillium crustaceum*, *P. brevicaule*, and *Aspergillus glaucus* also failed to grow on the latter acid. All 4 of these fungi, however, grew on both acids in the presence of dextrose or mannite. For a number of the fungi glycine, uric and hippuric acids were found to serve to a small extent as sources of both C and N. Extracts of several of the fungi each fermented uric and hippuric acids, showing that the process is enzymatic. His 1914 contributions may be summed up as follows. Using the same 10 fungi, he found urea, uric acid, hippuric acid, glycine, guanine, guanidine compounds, nitrites, nitrates, and $CaCN_2$ serviceable as N sources in the presence of cane sugar, the $CaCN_2$, however, only weakly. Uric acid, hippuric acid, glycine, and guanine in the presence of a mixture of mineral N sources (KNO_2, NH_4NO_3, and NH_4Cl) served as C sources, but urea, guanidine, and KSCN failed to do so.

Growing the fungi on a KNO_3-sucrose medium and at intervals testing for nitrites, Kossowicz obtained positive indications in all cases but very irregularly. Ammonia formation was evident in cultures of *Aspergillus niger* and *A. glaucus*, *Cladosporium herbarum*, *Penicillium glaucum*, and *Fusarium* sp., but the fact that he did not obtain NH_3 in 21 days' incubation, as shown by his seventh experiment, indicates autolysis as the cause for its presence. Distillation with MgO was used for NH_3 determination, 20 ml. of the culture solution being taken; the Gręiscz and the Zn-iodide-starch methods were employed for the qualitative nitrite test and Nessler's reagent for NH_3. Of 7 different yeasts tried the formation of nitrite from nitrate could not be established for any. Nitrate was found a poor source of N for yeasts.

Brenner ('11) gave neither his biochemical methods nor any tables of results in his paper dealing with the nutritive value to *Aspergillus niger* of 30 various N sources. He stated that the assimilability was judged by determining the time required for the cultures to reach a maximum weight. Not considering the C of the organic N compounds, dextrose was the sole C source employed. The temperature of incubation was 35° C. All N sources were used in the concentration equivalent to 0.5 per cent NH_4Cl. Free NH_3, $NaNO_2$, ammonium valerianate, and KCN were poisonous to this fungus in the strength used. Tetramethyl-ammonium chloride, nitroguanidine, nitromethane, isoamylacetate, pyridine chloride, and piperidine chloride were not assimilable. Four groups were made in the descending order of their assimilability:

1. Ammonium lactate, ammonium tartrate, asparagin, ammonium succinate, and ammonium oxalate.

2. NH_4 salts of H_2SO_4, HCl, HNO_3, and H_3PO_4, likewise carbamide.

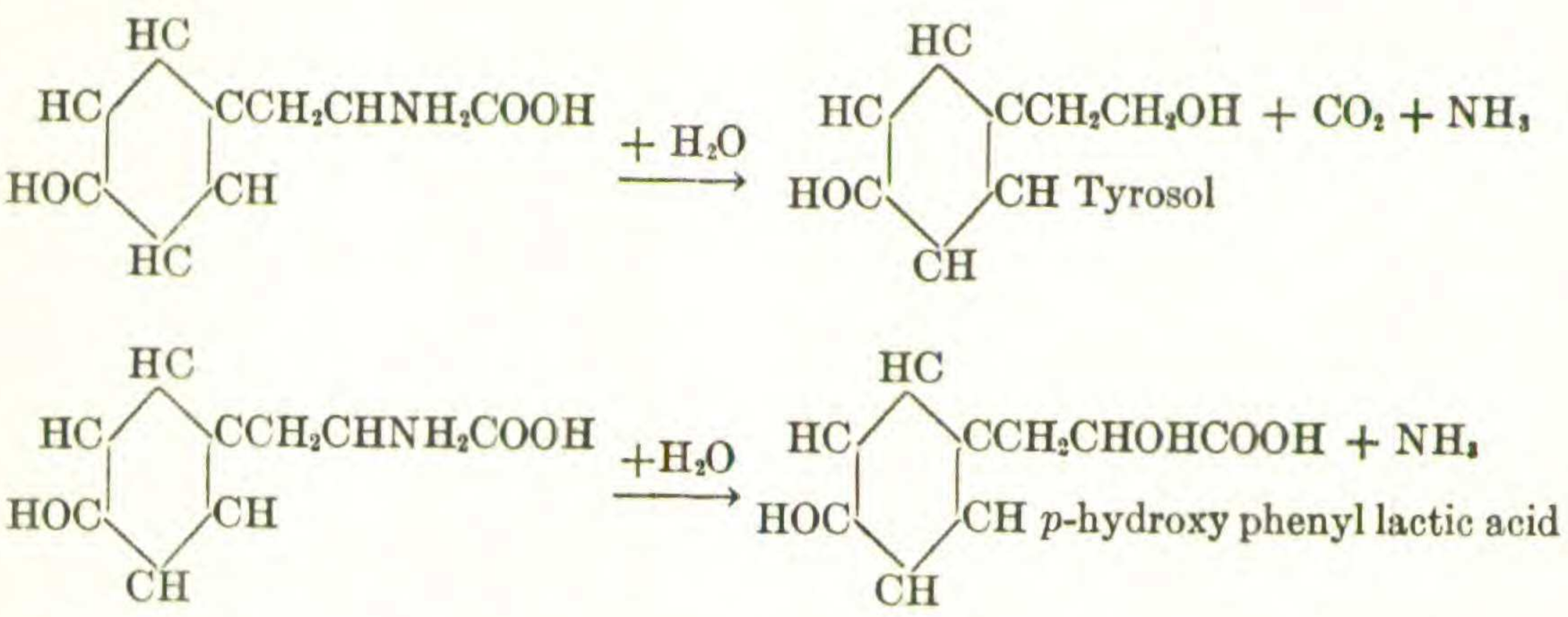
3. CH_3COONH_4, $HCOONH_4$, nitrosodimethylamine chloride, $NaNO_3$, pyridine nitrate, normal and isobutylamine chloride, guanidine nitrate, and chloride.

4. Isoamylamin chloride, hydroxylamine sulphate, benzylamine sulphate, dicyandiamid acetonitrile.

He stated that his study of the composition of the fungus and changes in the medium showed that after a growth period of about 4 days degenerative processes began in parts of the fungus. These were accompanied by the secretion of N as NH_3 or organic N. As a rule, regardless of the nature of the N source, about one-half of the N present in a solution containing the equivalent of a 0.5 per cent NH_4Cl solution was taken up by the first crop of the fungus grown. Subsequent crops, having less N at their disposal, contained a lower percentage of N than the first crops on the same solution.

A clearer conception of the course of protein synthesis in yeasts and other fungi is given by the illuminating qualitative and quantitative work of Ehrlich ('09, '11, '16, '17) and his associates. Following the lead of Duclaux ('64) and Pasteur ('58, '59) Ehrlich found that yeast could transform amino acids into alcohols

having one less C atom than the corresponding amino acids. Filamentous fungi, on the other hand, decomposed the amino acid to the corresponding hydroxy acid. Yeasts, especially *Willia anomala* and wild yeasts, were employed, as was *Oidium lactis*, *Rhizopus nigricans*, *Aspergillus*, and other fungi. Tyrosine, for example, was found to be hydrolytically decomposed by yeasts and fungi respectively, according to the following equations:



The technique by which the alcohol and hydroxy acid were isolated, identified, and quantitatively determined were given. The yield in some cases was almost quantitatively equivalent to the amount of amino acid consumed; that is, the N of the fungous mat determined by Kjeldahlization plus the non-nitrogenous complex, tyrosol, corresponded reasonably to the added amino acid, tyrosine. The ammonia formed is used up by the organism in its building of protoplasm and is not detectable in the culture fluid. The tyrosol produced in the presence of other carbon sources is not further used, but is really a by-product which diffuses out through the plasma membranes of the yeast cells.

Other amino acids were tried with similar results; for example, from tryptophane (β indolalanine) was obtained tryptophol (β indolethyl alcohol), a new compound whose properties were described in detail (Ehrlich, '12). Likewise from leucine and isoleucine yeasts formed amylalcohol and active amyl alcohol respectively. That the amino acids probably served solely as a source of N to the organism was indicated by the fact that upon the addition of ammonium salts to the medium the amino acids were not utilized. Boas and Leberle ('17), in this con-

nection, also found that *Aspergillus niger* utilizes only $(NH_4)_2SO_4$ as a source of N when both this and acetamide are present in the medium, and only glycine when this and acetamide form the N supply. They, moreover, found that $(NH_4)_2SO_4$ was used in preference to peptone in spite of the harmful effects of the acid freed in the utilization of the ammonia part of the $(NH_3)_2H_2SO_4$.

In Ehrlich's 1909 investigation yeasts were found to ferment glutamic acid to succinic acid; this was explained as an oxidation rather than an hydrolysis.

$$\begin{array}{l} CH_2COOH \\ | \\ CH_2 \\ | \\ CHNH_2COOH \end{array} \xrightarrow{+\,O_2} \begin{array}{l} CH_2COOH \\ | \\ CH_2COOH \end{array} + CO_2 + NH_3$$

Reactions that did not run as smoothly as these cited were explained by assuming that substances arising first were either unstable or suffered a change through secondary reactions.

The conclusion may be summed up in the words of the investigator:

"Nach den vorstehenden Ausführungen kann es keinem Zweifel unterliegen, dass auch bei der Assimilation von Aminosäuren durch Hefe der Aufbau ihres Plasmaeiweisses nicht anders erfolgt, also wenn der Hefezelle nur Ammoniak und Zucker dargeboten werden. Das die Hefe imstande ist, mit Ammoniak als alleiniger Stickstoffnahrung und Zucker also einzigem kohlenstoffhaltigem Material auszukommen und sich darauf normal fortzuentwickeln, haben ja bereits die Arbeiten Duclaux's einwanfrei erwiesen. Für den analogen Vorgang beim Wachstum auf Aminosäurenlösungen liess sich experimentell indirekt dadurch ein überzeugender Nachweis erbringen, dass es gelang, durch Zusatz von Ammonsalzen zu der gärenden Flüssigkeit auch im Überschuss vorhandene Aminosäure vor dem Angriff durch die Hefe zu schützen und dementsprechend die Menge der sonst auftretenden Abbauprodukte wie Fuselöl, Bernsteinsäure, Tyrosol u.s.w. auf ein Minimum zurückzudrangen. (See under "discussion," p. 350.)

Ehrlich and Pistschimuka ('12) extended this thought further to the utilization of primary amines by yeasts and fungi. Analogous to the fermentation of the amino acids, the primary amines underwent a similar decomposition to alcohols by hydrolysis of the amido group and splitting off of ammonia, which alone was used by the organisms.

$$RCH_2NH_2 + H_2O \longrightarrow RCH_2OH + NH_3$$

Tyrosol and fusel oil respectively were obtained from *p*-hydroxy-phenyl-ethylamine and isoamylamine. Guggenheim and Löeffler also showed this to take place in animal tissues.

Ehrlich ('16) then worked with the trimethylated amino acid, betaine, the secondary amine, adrenalin, and the tertiary amine, hordenin, and found the utilization of these compounds by fungi and yeasts analogous to the above. From betaine was obtained glycollic acid and trimethylamine, the latter being hydrolyzed by the organism to methyl alcohol and the directly usable ammonia. Adrenalin was hydrolyzed to *m-p*-dihydroxyl-phenyl-ethylene glycoll and monomethylamine; the poisonous hordenin, to the harmless tyrosol and dimethylamine, the amines in both cases, as with betaine, being further hydrolyzed to NH_3 and CH_3OH. In concluding, this investigator emphasized the use to which fungi may be put in the preparation of organic compounds otherwise difficult or impossible to prepare. He also pointed out the possibility that these reactions might throw light on the question whether the alkaloids of green plants, which are rich in alkylamines, are end products of metabolism or merely intermediate compounds which undergo a further change. He then emphasized the known relation of betaine and glycollic acid in sugar beets.

Completing the analogy, Ehrlich ('16) grew several organisms, including *Willia anomala*, *Oidium lactis*, *Pichia farinosa*, *Penicillium glaucum*, and *Aspergillus niger*, on varying concentrations of alkaloids in the presence of various quantities of alcohol, or of invert sugar as carbon sources. Among the alkaloids tried were: cocaine, brucine, morphine, chinchonine, and nicotine. Determining the dry weights of the organisms (where possible) and the N in the mat, and observing the odor and appearance of the culture, he came to the conclusion that compounds possessing an easily split-off N group, as the piperidine group, are more readily assimilable than others. Nicotine, for example, having the easily detached pyrrolidine ring, is better than brucine, morphine, and others in which the N group is held more closely. The molds and bacteria of mixed cultures utilized more of the alkaloids than did pure cultures of individual organisms.

Like Brenner ('11), Puriewitsch ('12) reverts to the view of Czapek that α-amino acids serve directly as materials for protein synthesis and are therefore the most favorable N sources. This investigator attempted to clarify the problem of protein synthesis by determining the energy required for the assimilation of the different nitrogenous compounds. The energy was estimated by measuring the CO_2 evolved per unit of dry weight of the fungus produced. *Aspergillus niger* was the organism employed. The CO_2 was swept out by a stream of air into tubes containing KOH and weighed. Used with dextrose, the amino acids, methyl urea, KSCN, acetamide, urea, and methylamine gave a low ratio $\frac{CO_2}{\text{dry wt.}}$; while KNO_3, ethylamine, phenylurea, guanidine, protein, and peptone gave higher ratios, showing that they required more energy for their assimilation. Ammonium salts occupied about a middle position. Some of the N compounds were also tried with malic, succinic, and tartaric acids as carbon sources in the place of glucose. Although the ratios obtained were somewhat higher than with the sugar, the same general order of assimilability of the N compounds was obtained. However, with malic acid the NH_4 salts were superior to glycine, and KNO_3 almost as good. With respect to the C sources the ratio increased in this order: dextrose, succinic acid, malic acid, and tartaric acid. It is difficult to understand the interpretation of Puriewitsch and his inclination towards Czapek's view, when one examines his data for peptone, which gave a high ratio for all C sources.

Dox and Maynard ('12) cultured *Aspergillus niger* and *Penicillium expansum* in a liquid medium having sucrose and ammonium acid tartrate as the C and N sources, and at the end of each week made determinations of the total and ammonia N of the medium. They found that both forms of N decreased rapidly during the first week of growth, and then during the next 5 weeks increased to a constant quantity. The N retained in the mycelium after this equilibrium had been established was thought to be "some chitin-like substance or glucosamine complex which does not undergo autolytic change." Similar results were obtained when KNO_3 was the N source. Dox ('13) continued this work with

A. niger, making qualitative tests for the presence of sugar and noting the effects on N excretion of replacing the medium weekly by water or by a 2 per cent sucrose solution. The determinations showed that autolysis of this fungus is largely due to the exhaustion of carbohydrate from the medium, because a removal of the autolytic products and substitution of distilled H_2O increased the rate of autolysis, and replacement of the culture solution by sugar solution lessened the rate to one-half that of undisturbed cultures and to one-third that of the cultures in which distilled H_2O replaced the medium.

Waterman ('13), by shaking for 2 hours, a dried, living mat of *Aspergillus niger* with a nutrient solution containing 2 per cent glucose, removing and washing the mat, then boiling for 10 minutes in H_2O and testing the extract with Fehling's, showed that no glucose as such had entered the mold. This was interpreted as showing that the protoplasm of the mold behaved as a semi-permeable membrane toward the glucose, and that adsorption was of no consequence in the accumulation of nutrients. His tables of data show that he determined qualitatively with Nessler's reagent and diphenylamin-sulphuric acid the ammonia present or developed in the culture solution; in some instances this was determined quantitatively with the same reagents. The total nitrogen fixed in the mold and the percentage of sugar assimilated were determined quantitatively, but the methods used were not described. It is assumed that Kjeldahlization, for the N, and some method with Fehling's solution, for the glucose, were employed. Waterman believed that his results showed that "ammonia is a normal excretion product in the metabolism of *Aspergillus niger*" and that this fungus "is able to reduce nitrate to ammonia." A young culture, in which the N content was 2 to 2½ times that of a mature mat, was thoroughly washed and the fungous mat then boiled in water. The extract contained no trace of the inorganic salts originally added to the nutrient, showing that the N salt assimilated by the fungus was quickly changed into another form, and adsorption has little influence on the nutrition. Waterman's other results are given in his own words.

"1. The nitrogen fixed in the mature mould is proportional to the

plastic equivalent of the carbon independently of the nature of the carbon as well as of that of the nitrogen.

"2. The nitrogen number, by which is meant the nitrogen per 100 parts of weight of assimilated carbon, lowers with time; for a mature mould it is about 2 (glucose or levulose as source of carbon).

"3. The metabolism of nitrogen has much resemblance to that of the carbon.

"a. An accumulation of carbon is combined with a high nitrogen number; inversely the mature mould has a low nitrogen number.

"b. The nature of the metabolism of the nitrogen does not change under the influence of many factors; neither is this the case with the carbon.

"c. The velocity of the metabolism is subject to great changes.

"d. The same factors that accelerate the metabolism of the carbon also furthers that of the nitrogen.

"e. Substitution of rubidium for potassium is of little influence on the metabolism of nitrogen.

"4. The nature of the metabolism of the nitrogen is independent of the source of nitrogen. At first the nitrogen number is high, then it decreases whilst the freed nitrogen returns into the nutrient solution as ammonia. This is proved for the cases when ammonium nitrate, ammonium chlorid, or potassium nitrate, is given as N food. *Aspergillus niger*, thus, reduces nitrates to ammonia but not to free nitrogen. Only in the culture tubes with a deficiency of nitrogen as to the quantity of carbon, no ammonia can return into the solution as it is directly used for the production of new cells.

"5. In cases of a deficiency of N no fixation of atmospheric nitrogen could be observed."

Zaleski and Israilsky ('14), working with yeasts, found that single amino acids stood below NH_4 salts in point of assimilability. This, they explained, was comprehensible when one considered that yeast cannot build protein directly out of a single amino acid, but must first deamidize it to obtain N for other groups of the protein molecule. Asparagin was superior to NH_4 salts because the acid amide part of the molecule is readily deamidized and the N group thus obtained is first used in the formation of amino acids and then coupled directly with the liberated aspartic acid to form the protein chain. Aspartic acid and NH_4 salts mixed gave as good growth as asparagin; but, as shown by the fact that the amide group of the asparagin protected added NH_4 salts, the amide group was found superior to NH_4. The best source of N was that found in autolyzed yeast because, they explained, the suitable amino acids were linked directly to form protein. Where asparagin was added to

the autolysate it was found that yeast consumes only about 20 per cent of the amide group, whereas 80 per cent of the amino acid part was used in the synthesis. The description of the methods employed is very indefinite. The fermenting fluid was shaken to obtain a homogeneous mixture and then pipetted off. Protein N was estimated by Stutzer's method and by precipitation with iron acetate. In experiments 5 to 10 total and NH_4.N were determined and in experiment 11 the N of the amide group, but the methods are not given. The work of Ehrlich was criticized on the basis of the absence of NH_3 in the medium.

In an interesting investigation with *Aspergillus niger* in which $(NH_4)_2SO_4$ was used in conjunction with various amino acids, peptone, autolysate, and amino acid mixtures, Zaleski and Pjukow ('14), by determining the total and NH_3.N of the culture fluid, obtained satisfactory results. Knowing the original N they computed from these determinations the kind and amount of N consumed. $(NH_4)_2SO_4$ was utilized to a greater extent than single amino acids in mixtures of the two, but the NH_4 salt, as shown by consumption, was not so good as mixtures of the amino acids or the fungous autolysate, when mixed with these organic sources of N. The order of the serviceability of single amino acids used with $(NH_4)_2SO_4$ was phenylamine, leucine, glycine, alanine, aspartic acid; histidine was not utilized. The fact that $(NH_4)_2SO_4$ was used even in the amino acid mixtures showed that the fungus synthesized other amino acids. The decomposition of the various amino acids proceeded at different rates which were proportional to their utility for this organism. The relative usefulness of the NH_4 salts and amino acids could be varied in several ways: (1) by varying the acid radical to which the (NH_4) was joined, (2) by changing the carbon source; with a less available carbon source the amino acid was more utilized, (3) by the addition of a stimulant, as $ZnSO_4$, which lessened the consumption of the sugar. Glycine in the presence of CH_3. $COONH_4$ was not used. The addition of $CaCO_3$ to the nutrient containing $(NH_4)_2SO_4$ and alanine decreased the use of the alanine, and this was explained by saying that the carbonate neutralized the acid produced in growth and produced an alkaline

reaction adverse to the decomposition of the amino acid. In the presence of a good C source NH_4 was found a better N source for the fungus than single amino acids, but a suitable mixture of amino acids was better than NH_4.

In his severe criticism of the opinions of Czapek, Puriewitsch, Brenner, and others on the direct usability of amino acids, Boas ('18) pointed out the fallacy of attempting to judge the comparative assimilability of N nutrients under the widely varying conditions of experimentation employed by those workers. For example, a hydrochloride of guanidine could not satisfactorily be compared with an amino acid because of the liberation from the former under the action of a fungus of an abundance of the inhibiting acid, HCl. He emphasized the necessity for comparing compounds at the same hydrion concentration; for example, in the comparison of asparagin and $(NH_4)_2SO_4$, the production of soluble starch and delay in sporulation observed with the latter compound, and which indicated an abnormal metabolism, resulted from or was conditioned by the liberation of H_2SO_4. Czapek, in his choice of incubation periods of 21 days or longer, it was pointed out, entirely ignored the effects of proteolysis on the fungous crops. Boas then repeated Czapek's experiment with guanidine, urea, and biuret, making weighings of the fungous crop, at different time intervals and obtained a reversal of the order of assimilability. The free acid liberated from the guanidine-HCl, it was thought, inhibited autolysis so that a long incubation period eventually gave a larger crop. Puriewitsch was also criticized on the same basis. Boas explained that the energy quotient would be a valuable index of the assimilability of N compounds, if due regard were given to the use of a variety of C sources, to the hydrion concentration of the medium, to the formation of soluble starch, to the effects of proteolysis, and to the influence of outer conditions, as temperature, light, humidity, and aeration. He then gave corrective experiments. Mannite, glycerine, malic acid, and quinic acid were employed as C sources, because, as he showed, the liberation of acid from the ammonium salts of the strong acids did not upset the carbon metabolism of the fungus when these compounds were used; soluble starch was not formed and there was no delay

in fruiting. In connection with these carbon compounds referred to, $(NH_4)_2SO_4$ and $(NH_4)_3PO_4$ were found superior to asparagin and glycine as N sources. The energy requirements for the amino acids were higher because of the necessary deamidization. The energy quotient for asparagin was raised by the addition of free H_2SO_4 to the culture fluid.

In his 1919 work Boas showed also that the rate of absorption of a compound was proportional to its extent of dissociation; for example, the highly dissociated NH_4 salts of strong mineral acids were utilized before glycine, acid amides, and peptones. This relation held in spite of strong acid formation and the consequent production of soluble starch and inhibition of sporulation. His 1922 article gave data showing that on media containing respectively, levulose, sucrose, dextrose, maltose, and galactose, decreasingly in order of the sugars listed, *Aspergillus niger* was capable of forming soluble starch. There appeared to be a close connection between diastase formation, sporulation, and hydrion concentration. In contrast to the behavior of *Aspergillus niger*, *A. Oryzae* on maltose caused soluble starch formation but on levulose produced none.

Waksman ('18, '19, '20), in his extensive studies with soil organisms, particularly the *Actinomyces*, gave due attention to the nitrogen relations. His 1918 work with several species of *Aspergillus*, *Citromyces*, *Penicillium*, and *Actinomyces*, and *Bacterium mycoides* showed that such rapidly growing molds as *Aspergillus* and *Penicillium* on Czapek's solution, with peptone or casein as the N source, produced an abundance of ammonia which gradually increased with the duration of incubation; amino nitrogen, on the other hand, tended to decrease, indicating use by the organisms. The slower-growing *Actinomyces* and *Bacterium mycoides* brought about a large accumulation of amino nitrogen and a relatively small accumulation of ammonia. Another group, represented by *Citromyces* spp., favored the accumulation of both forms of N in relatively large amounts. In the work with *Aspergillus niger* it was shown that this accumulation of NH_3 took place both in the absence and presence of sugar, but the sugar depressed the rate of production. In the presence of sugar the curve for NH_3 accumulation followed remarkably the

theoretical curve for autocatalysis. The relation of ammonia production to carbohydrate present was further shown by the culture of *A. niger* on media containing varying percentages of asparagin and the same concentration of sugar. On media having 0.5, 1.0, and 2.5 per cent asparagin the organism after reaching its maximum growth (in about 3 days) rapidly decomposed the amide-amino acid into ammonia nitrogen; on the other hand, where only little asparagin was present and the amount of sugar remained the same, that is, relatively large in comparison, little or no ammonia was produced and the amount of NH_3.N simply decreased.

Making further use of the van Slyke "micro" method for amino N, Waksman ('18) made a study of the proteolytic enzymes of several soil fungi, *Aspergillus niger* being employed in most of the work. He showed that *Penicillium chrysogenum* and *Actinomyces* sp. 101, which had been found to favor the production of more amino than ammonia N when grown in a peptone solution, produced strongly proteolytic enzymes, whereas the organisms, as several species of *Aspergillus*, which produced more ammonia than amino N, formed weaker enzymes. Compared with the proteolytic enzymes of animal origin, the fungous enzymes were found to differ in being less limited by hydrion concentration (P_H values were not determined; reactions were indicated by litmus and phenolphthalein), by having a lower optimum temperature, by not being precipitated by safranin, and in being able to pass a Pasteur-Chamberland filter. In its production of enzymes *Aspergillus niger* was not influenced by the sugar content of the medium. The activity of both exo- and endo-proteoclastic enzymes produced by the fungi in peptone-containing media was greater than that of the enzymes produced in nutrients containing no peptone. The fast-growing *Aspergillus niger* produced its most active enzymes during the first 3 days of incubation, whereas the slower-growing *A. ochraceus* increased in proteoclastic activity up to the eighteenth day of incubation, after which there was a decline. He attempted an explanation on the grounds that *A. niger*, reaching its maximum growth in about 3 days, during this time necessarily produced strongly acting enzymes and also acids, which acted injuriously

on the enzymes. The greatest enzymatic activity for *A. niger* was therefore observed during the first 3 days of incubation. *Aspergillus ochraceus*, on the other hand, was considered to produce no injurious acids, and its enzymes increased in activity until the inhibiting autolytic substances began to be formed. The exo- and endo-enzymes were similar in their action. Deamidizing enzymes were indicated by the detection of small amounts of ammonia in the treated peptone and casein.

In the work on the N metabolism of the *Actinomyces* Waksman and Curtis ('16) and Waksman ('18, '19, '20) made use of Nessler's reagent for qualitative ammonia tests and the aeration method for quantitative determinations; amino N was estimated by the "micro" method of van Slyke, nitrites by the colorimetric method of Griescz, and active acidity with Clark and Lub's indicators. The results may be summed thus. Nearly all *Actinomycetes* were capable of liquefying gelatine and many could haemolyze blood agar and liquefy blood serum; in other words many of these forms have a strong proteoclastic power. This process was followed in protein solutions by means of amino acid determinations. The amount of splitting was directly proportional to the extent of growth. Although on long incubation a considerable quantity of NH_3 accumulated, the production of NH_3 from amino acids and proteins by *Actinomyces* was not considered characteristic. None could fix elementary N_2. With a suitable C source nearly all species could reduce nitrates to nitrites. In the order of assimilability as sources of N stood, speaking broadly, proteins and amino acids, nitrates, nitrites, amides. With glycerine as a carbon source NH_4 salts were the poorest sources of N; but with dextrose both NH_4 salts and amides were well assimilated if the medium did not become too acid.

Waksman and Joffe ('19) reviewed some of the papers on the application of hydrion determinations to bacteriological work; they then gave the results of investigations on the effect of *Actinomycetes* on the reaction of Czapek's solution containing various C and N sources. The colorimetric method was used. It was shown by growing the organisms on $NaNO_3$ and varying the C source that the *Actinomycetes*, unlike many bacteria, are not producers of acid from carbohydrates; in fact under the con-

ditions the medium showed a tendency toward alkalinity. His explanation was that the nitrate, under the influence of these organisms, is reduced to nitrite and the liberated oxygen is united with the reducing H to form hydroxyl ions which reduce the hydrion concentration of the medium. With the conditions reversed, employing constant C source (glycerine) and different N sources, the change in the reaction of the medium was shown to be due to the N source, $(NH_4)_2SO_4$ medium, for example, rising in hydrion concentration from P_H 5.8 to 4.2. The change of P_H in protein and amino acid media was found very variable, depending on the species of the organism cultured, the original P_H, and the C source. An available C source in a protein medium favored the production of an acid reaction; this was thought to be due to the effect of the carbohydrate on the N metabolism and not to the formation of acids from the C source. Some species always increased the $[\overset{+}{H}]$ in such media, while others lessened it. Leucine nearly always favored acidity. The $[\overset{+}{H}]$ of all media was shifted toward the optimum by these organisms.

Molliard ('18) studied the rate of consumption by *Sterigmatocystis nigra* of glucose and levulose, resulting from the inversion of sucrose, in a modified Raulin's solution. Sucrose was added with HCl in 2 different concentrations (290 mgm. and 310 mgm. HCl per 150 ml. of medium) for the 2 series run. The increase in acidity lowered the harvest of fungus, but increased the consumption of sugar. The determinations made at 21 days showed that all the glucose, but only about one-sixth of the levulose, had disappeared. In his 1920 work on the effect of reaction on the liberation of CO_2 from cultures of the same fungus Molliard varied the acidity with H_2SO_4 and Na_2CO_3, and recorded the reaction in terms of normality of acidity or alkalinity. Determinations of active acidity were not made. It was found that the amount of CO_2 set free in respiration increased rapidly from 0.1 *N* alkalinity to a maximum at 0.02 *N* alkalinity, then diminished slowly to 0.06 *N* alkalinity,—beyond this very rapidly. Oxalic acid was said not to be produced in the acidity range greater than 0.02 *N* H_2SO_4, but steadily increased in production as the medium was made more alkaline, reaching a maximum at 0.06 *N* alkalinity.

Raistrick and Clark ('19) determined the relation of various carbon sources (organic acids) to oxalic acid formation by *Aspergillus niger*. Oxalic acid was determined by precipitation as calcium oxalate and titration with permanganate. The results are summarized:

1. Four carbon, dibasic acids gave good growth and a good yield of $(COOH)_2$.

2. Four carbon, monobasic acids gave almost no growth and $(COOH)_2$.

3. Three carbon acids gave very good growth, but little or no $(COOH)_2$.

4. Two carbon acids, as acetic, gave good growth and yield of $(COOH)_2$, while glycollic and glyoxalic gave but fair growth and no $(COOH)_2$.

5. The one carbon acid, HCOOH, gave but fair growth and no oxalic acid.

A theory was given to account for the formation of $(COOH)_2$, citric, and fumaric acids from sugars from other organic acids. In the case of $(COOH)_2$ production from sugar, diketo adipic acid is formed which is hydrolyzed to acetic and oxalic acids, the CH_3COOH then being also oxidized to $(COOH)_2$. With the organic acids as C sources, oxalacetic acid is formed by hydrolysis or oxidation or both, depending upon the organic acid used, and this breaks to form $(COOH)_2$ and CH_3COOH. The production of citric and fumaric acids from sugar was supposed to proceed through oxalacetic acid. The sources of the inorganic N (NH_4 or NO_3 ions) were found to have no influence on the quantity of $(COOH)_2$ formed.

Lampitt ('19) presented data relative to the N metabolism of bread yeast. He determined total N by Kjeldahlization, NH_3 by distillation with MgO *in vacuo*, and sugar by Bertrand's method. Deamidization of amino acids was not studied by determinations of amino nitrogen but by the ammonia produced. It was found that the removal of N from the nutrient was proportional to the yeast present; and, as determined by counts and N determinations, the greater the rapidity of budding during active fermentation the greater the amount of N assimilated by each cell. However, active reproduction was found in some

cases to result in a lowering of the N coefficient of the yeast, even in the presence of an abundance of N. The final N coefficient of the yeast was found to be independent of the initial coefficient for the particular conditions of reproduction.

A non-volatile acid, thought to be malic, resulted from the action of yeast on asparagin. Malic acid itself was not fermentable; but its NH_4 salt was entirely decomposed, producing C_2H_5OH. Propionic acid, which, according to Effront, results from the action of amidases on asparagin, was not fermented and its NH_4 salt only slightly attacked. Fermentation was found to be necessary to the assimilation of N, yet the two processes were not proportional, for deamidization was retarded during excessive zymatic action and sometimes continued after this had ceased. Excretion of N into the medium also was found to be conditioned by fermentation, but not proportional to it; it was, however, directly proportional to the sugar present. The process took place simultaneously with N assimilation, indicating that excretion was interrelated with the life of the cell; and the N excreted was shown to be utilizable under certain conditions.

Iwanoff ('21) pointed out the defects of the Stutzer method, as used in the determination of the protein N, of fermenting fluids; nitrogenous bodies having no protein character were found to be precipitated by the $Cu(OH)_2$. These bodies were huminose in character and resulted from the reaction of sugar with an N-containing substance coming from the yeast. These huminose compounds plus alcohol and the other products of fermentation arrested protein decomposition more than did alcohol of the strength present in the fermenting liquid, but it was thought that the acids present were largely responsible for the difference. Alcohol, however, was found to be the chief inhibitor of all the fermentation products, 7 per cent strongly inhibiting the protein decomposition. The addition of KH_2PO_4 tended to annul this. As a possible explanation of Iwanoff's huminose compounds Gortner and Holm ('17) have shown that the dark nitrogenous substance, "humin" N, formed during the acid hydrolysis of proteins is due to the action of an aldehyde on the indole group of tryptophane.

Haenseler ('21) found that the yield in dry weight of *Aspergillus*

niger was proportional to the amount of nitrate and also to the concentration of the sugar of the culture medium.

The action of nitrate on lower organisms was studied by Böttger ('21). Toxicity appeared at a certain concentration of the nitrate, above which it became increasingly inhibitive until the organisms were killed. The initial point of toxicity varied with the other components of the nutrient. The different functions, such as growth, enzymatic activity, and sporulation, exhibited toxic response at different concentrations of the nitrate. The specific nature of the toxic property was not determined, but it was thought to be nutritive as well as physical (osmosis). All nitrates were found toxic regardless of the cation. Waterman ('18) gave a series of tables indicating the influence of KNO_3 on the rapidity of growth of *Aspergillus glaucus*. Kossowicz ('12) demonstrated the poisonous effect of $CaCN_2$ on 10 different fungi.

Self-poisoning of fungi might be briefly mentioned here, as the process is closely related to N metabolism. Uhlenhaut ('11) grew species of *Mucor* on media containing the glucoside, amygdalin, and found that growth was soon inhibited. This inhibition was ascribed to the accumulation of benzene cyanhydrine which imparted an easily recognizable odor to the solution. Where fungi capable of utilizing cyanhydrine were cultivated with *Mucor*, the latter made a much more abundant growth than in pure culture. Amygdalin was little used in the presence of other C sources. Wehmer ('13) noted self-poisoning of *Penicillium variabile* on media with $(NH_4)_2SO_4$ as the N source. Boas ('19), in a former article, had shown that a *Cladosporium* in media having urea as the N source produced such large quantities of NH_3 that the fungus was soon killed; amines, produced proteolytically, were also thought to be instrumental in the death of the fungus. *Aspergillus niger*, on a solution containing 2 per cent urea, 5 per cent maltose, and the usual mineral salts, quickly changed the reaction of the medium to strongly alkaline (P_H 7.5–8.3). A strong odor of ammonia and amines was evident, the NH_3 neutralizing the oxalic acid produced from the sugar. The fungus was killed in from 7 to 9 days on this medium, as it was also on that containing various amounts of maltose, dextrose plus

maltose, and sucrose with urea and acetamide in different proportions. Other fungi, including *Botrytis cinerea* and an *Oidium*, behaved quite differently under these conditions, remaining alive for months and not producing excess NH_3.

Terroine, Wurmser, and Montaine ('22) investigated the total N content of *Aspergillus niger* grown under various conditions. With the development of the fungus the N percentage of the mat was found to decrease; this was not influenced by the concentration of the N of the medium. In the first part of the incubation, when the N content of the medium was 0.5 per cent $(NH_4)_2SO_4$, an increase in the sucrose or glucose was followed by an increase in the N fixed; in the latter part, by a decrease. Urea or $NaNO_3$ substituted for $(NH_4)_2SO_4$ resulted in a slight fall in the percentage of fungous N, but peptone and guanidine caused a decline respectively of 18.3 and 45.0 per cent. Xylose and arabinose with $(NH_4)_2SO_4$ served in this respect exactly as glucose or sucrose, but galactose caused a reduction of 21 per cent. The mycelium of a normal culture washed and placed in Czapek's solution minus N lost over 50 per cent of its N in 5 days' incubation at 37° C.

Butkewitsch ('22) grew *Aspergillus niger* and *Citromyces* spp. on culture solutions containing the usual minerals plus 0.2 per cent $ZnSO_4$ and respectively 2.5, 5.0, 10.0, and 20.0 per cent peptone and determined the oxalic acid, the ammonia, and likewise the dry weight of fungus produced in 10-, 20-, 30-, and 40-day periods of incubation. Hydrion concentration determinations were not made, litmus being employed to indicate the reaction. Ammonia was estimated by distillation with MgO or CaO *in vacuo*, and $(COOH)_2$ by precipitation as calcium oxalate and titration with $KMnO_4$. The results were interpreted as showing that the proportion of $(COOH)_2$ to NH_3 approached that of neutral $(COONH_4)_2$, but generally showing a predominance of NH_3. The younger cultures were acid because in addition to $(COOH)_2$ they contained the acids freed in the deamidization of the amino acids by the fungus; the older cultures were alkaline because of the excess NH_3 not bound with acids, but as $(NH_4)_2CO_3$. Most of the NH_3 was produced in the period of mat development, 90 per cent of it being formed in the first

10 days; the ratio $\frac{\text{dry wt.}}{NH_3.N}$ was consequently greatest in this period and decreased as the culture aged. This diminution of the ratio $\frac{\text{dry wt.}}{NH_3.N}$ was found to correspond to the peptone content of the medium; the greater the initial growth the greater the ultimate decrease in weight. Rise of temperature decreased this fraction. Age and temperature determined also the economic coefficient of the N-free complex of the peptone. Zinc salts had no effect on the utilization of peptone, so that the increase in harvest obtained with media containing carbohydrate as a C source to which Zn had been added was explained as due to stimulation of carbon metabolism. *Citromyces* behaved similarly to *Aspergillus*, but produced a slightly higher percentage of NH_3.

Boncquet ('16) found nitrites and NH_3 in material from "mosaic" tobacco and potato, and from beets affected with "curly leaf." He ascribed the presence of these unusual nitrogenous forms to the "reducing power of the internal bacterial flora." Jodidi, Moulton, and Markley ('20) ran parallel nitrogen analyses with normal and "mosaic" spinach and found remarkable differences. Diseased plants had a smaller percentage of total protein, nitrate, acid amide, mono- and di-amino N, but a slightly higher percentage of NH_3 than normal plants; nitrites were found only in the diseased plants. Obtaining similar results with material from normal cabbage and that affected by the so-called "mosaic disease" they believed they were warranted in cataloging the disease as "mosaic." Klotz ('21), using the same methods, obtained similar analytical data working with celery leaves affected with the *Cercospora* blight; later it was found that celery leaves diseased with *Septoria* blight showed a similar chemical picture. In the latter case more modern methods were used.

EXPERIMENTAL

MATERIALS AND METHODS

Three fungi, viz., *Aspergillus niger*, *Sphaeropsis malorum*, and *Diplodia natalensis* were used in the investigations reported in this paper. Work with *Phoma Betae*, a form particularly in-

teresting because of its capacity for fixing free N_2 (Duggar and Davis, '16), is now under way. The investigation of other forms, including yeasts and bacteria, is contemplated.

The organisms were grown on Duggar's solution for fungi,[1] the nitrogen source being varied to give 5 different kinds of media. The solution consisted of the following chemicals per 50 ml.

		ml.
Dextrose	0.5 *M* solution	25
KH_2PO_4	0.25 *M* solution	10
$MgSO_4.7H_2O$	0.1 *M* solution	5
N source	*M* solution	10

In addition to the above 0.1 ml. of 0.001 *M* $FeCl_3.6H_2O$ was added to each culture. The KH_2PO_4, $MgSO_4.7H_2O$, and $FeCl_3.6H_2O$ were Merck's highest purity grade. The inorganic N sources used were KNO_3 (Merck's reagent crystals), NH_4NO_3 (H. P. Merck), and $(NH_4)_2SO_4$ (Merck's reagent grade). The salts were dissolved in warm distilled water (P_H 5.3), cooled to 20° C., made to volume, placed at a temperature of 7° C. for 3 days, and filtered. The stock solutions were kept in the dark at a temperature of 7° C.; before use they were brought to room temperature (20–23° C.). "Bacto" dextrose, having less than 1.0 per cent H_2O, usually about 0.25 per cent, was used. "Bacto" peptone was used in the concentration of 11.65 per cent throughout the work. The glucose and peptone solutions were made up just before using, thus necessitating but one sterilization which, for the peptone, was important in keeping comparable the amount of hydrolysis. The 5 different media are characterized in the data by the N source, namely, "peptone plus" (No. 1), "peptone minus" (No. 2), $(NH_4)_2SO_4$ (No. 3), NH_4NO_3 (No. 4), and KNO_3 (No. 5). The media numbered 1, 3, 4, and 5 have glucose as a carbon source, but in No. 2 the peptone serves as the source of both C and N, the 25 ml. of dextrose being replaced by 25 ml. H_2O.

Quantities of 50 ml. of the media were placed in 300 ml. Erlenmeyer flasks. The flasks were in all cases first thoroughly cleaned by washing in warm tap-water and cleaning solution, followed by several rinsings of hot tap-water and finally with distilled water.

[1] Being published.

For sterilization the media were autoclaved at 16 pounds steam pressure for 15 minutes. The sterile flasks were carried from the autoclave immediately to a thoroughly steamed culture room and inoculated when cool.

Three methods of inoculation were used in the course of the work. In the first and second series with *Aspergillus niger* plantings of the fungus were effected by wetting a platinum loop in the sterile media, placing it on the aerial spores of a potato glucose agar slant culture, and then transferring the loopful of spores to the medium. In the third series with this fungus a spore suspension was made by pouring 10 ml. of sterile distilled water on an agar slant culture, loosening the spores into the H_2O by means of a platinum needle and pouring the suspension into 100 ml. of H_2O in an Erlenmeyer flask. The flask was thoroughly shaken to obtain a uniform distribution of the dilute suspension and let stand over night to aid in wetting the spores. After shaking again the inoculum was taken up by means of a 25-ml. graduated pipette and 0.5-ml. portions placed into each flask of medium. With the *Sphaeropsis* and *Diplodia*, forms which did not sporulate in culture, uniform inoculations were obtained by planting the culture media with small (5 mm.) discs of inoculum from giant petri dish cultures on a thin (2 mm.) layer of potato-glucose (or sucrose) agar. As checks, several flasks of each medium were planted immediately before sterilization with the same amount of inoculum. All cultures in all series were incubated at 28° C. in darkness, a large incubator with double doors and water-jacket being used.

At the intervals shown in the following tables 5 cultures of each medium were analyzed, determinations being made of the dry weight and percentage N of the fungous crop, and hydrion concentration, sugar, NH_3 plus NH_4.N, total N, NO_3.N, NO_2.N, total amino N, acid amide N, and peptid N of the culture fluid. Near the end of each experiment entire cultures, including the mat and medium, were Kjeldahlized to ascertain the loss or gain of N and the presence or absence of the capacity of the fungi for N fixation under the conditions employed. The 5 culture solutions of each medium were filtered into a 500-ml. volumetric flask and the mats thoroughly washed, the wash water being

collected in the volumetric flask with the culture solutions. Aliquot portions of the filtrate and washings, which were made to volume and thoroughly mixed, were used for the determinations enumerated above.

The fungous mats were dried at 100–105° C. in an electric oven, cooled in a desiccator, and weighed to the nearest milligram on a "chainomatic" balance.

The active acidity of the culture solution was determined colorimetrically by employing the indicators and buffer solutions suggested by Clark and Lubs ('17) and Clark ('20). A comparator blank was used throughout. Near the ends of the indcator ranges it was helpful to make use of the colorimeter (Duggar and Dodge, '19), as this instrument extends the range and usefulness of an indicator. The micro Duboscq instrument can be applied very satisfactorily to this work by following these measurements. Two ml. of unknown plus 2 drops of indicator should be placed in the lower right cup, and in the cylinder above this .625 ml. of H_2O. In the left cup and cylinder should be placed respectively the corresponding quantities of known buffer plus indicator and compensating unknown solution. The readings are made at the 16.5th graduation in order to obtain like columns of colored solution and compensating solution.

Various methods for determining reducing sugars were tried. The results reported in the first series with *Aspergillus niger* were obtained by direct titration of the culture solution against Fehling's solution, the various indicators used to determine the absence of Cu and end point of the titration being tried. The 1920 iodometric method of Shaffer and Hartmann ('20) was found most satisfactory as it is accurate and rapid. The Fehling's modification was used throughout for "macro" quantities of sugar, as closer checks could be obtained with this than with the carbonate-citrate reagent. The advantage of the latter in having the chemicals combined in a single solution is outweighed by the increased cost and by the danger of loss of material from foaming when the solution is acidified at the end of the reduction. The "micro" method described in that paper was used in the second and third series with *Aspergillus niger* when the concentration of the glucose became sufficiently low; it was used in

subsequent series simply to ascertain the absence of the reducing sugar. Applied to the determination of dextrose in such media the "micro" method was found to give such variable results at different dilutions that it was entirely unsatisfactory for general use. Many of the following, very helpful suggestions were obtained from members of the staff at the Washington University Biochemical Department and are here given because they are not included in the paper. The "iodate-iodide" solution should be made slightly alkaline by the addition per liter of 0.4 ml. saturated NaOH solution; this prevents the formation of hydriodic acid. The standard thiosulphate solution is made permanent in the same way.

The thiosulphate solution is readily standardized by titration against a 0.1 *N* potassium biniodate solution (93.24958 gm. $KH(IO_3)_2$ per liter make 0.1 normality). To 50 ml. of the biniodate are added 3 gm. KI (dissolved in 25 ml. H_2O), and 10 ml. of an approximately 5 *N* H_2SO_4 or HCl. The excess acid and iodide with the biniodate liberate iodine equivalent to exactly 50 ml. of a 0.1 *N* solution, according to the following equation.

$$KH(IO_3)_2 + 10\,KI + 11\,HCl \rightarrow 6\,I_2 + 11\,KCl + 6\,H_2O$$

Titrate against the thiosulphate, using 2–3 ml. of starch solution as an indicator when the iodine color becomes faint.

Starch indicator solution made from arrowroot starch is preferable to a solution made from soluble starch because the latter deteriorates more rapidly under septic conditions and then fails to give the starch-iodine blue. Arrowroot starch solution kept for several weeks still retained its usefulness as an indicator. Of course any starch may be used, but the arrowroot is preferable because it is a standard, easily obtainable product. Two grams of the starch were shaken in 100 ml. cold water and poured into 100 ml. of boiling water; one ml. of 5 *N* H_2SO_4 was added and boiled about a minute.

The reduction is most uniformly effected by heating over a direct flame; an asbestos mat with a 2-inch circular hole is serviceable. A carrier, very handy for removing the hot flasks, is easily made by cutting away the side of a small, cylindrical wire test-tube basket, making an opening large enough to admit a 300-ml. Erlenmeyer. To cool, flasks are set in a shallow dish, as an evaporation dish, under running water.

The small quantity of reducing substances, other than dextrose, found in the peptone media could not be satisfactorily removed by precipitation with sodium tungstate as described for milk in Shaffer & Hartmann's ('20) paper. Lloyd's reagent,[1] as employed by Folin and Bergland ('22) (in the determination of sugars in urine), would adsorb some but not all of these reducing substances. Accordingly, in the later determinations the small quantity of copper reduced by the peptone was estimated by running blank determinations on the "peptone minus" (No. 2) medium, and subtracting this result from that given by the "peptone plus" (No. 1) medium.

The principle of the Folin ('05) aeration method was used in obtaining the results for ammonia nitrogen (see Shaffer, '03). To make the solution strongly alkaline to phenolphthalein 0.1 ml. of a saturated NaOH solution was used for each 5 ml. of unknown. The strong alkali is preferable to Na_2CO_3 because it assures the freeing of the NH_3 from the small amount of $MgNH_4PO_4.6H_2O$ that may form in the alkaline solution (see page 347). Tubes were connected in series so that 7 aerations could be made at the same time. Folin tubes with perforated bulbs were used throughout the apparatus for aerating the unknown and for delivery of the NH_3 into the receiving liquid. By means of a filter pump the solutions were aerated for 18–24 hours at the rate of approximately 50 liters of air per hour. In this, as well as in the determinations for total and nitrate N, 4 per cent boric acid was used to collect the NH_3. As in the Scales and Harrison ('20) method for total N the collected NH_3 was titrated directly against standard H_2SO_4, brom-phenol-blue being used as the indicator. This was compared with the usual method of collection in a standardized acid and titration back against standard alkali; the boric acid modification is absolutely as accurate as the old method and dispenses with the necessity of a standard alkali (see also Paul and Berry, '21, and Spears, '21).

Total N, including nitrates, was estimated by the method of Davisson and Parsons ('19). In this the nitrates are first reduced to NH_3 by means of Devarda's alloy in alkaline solution, the NH_3 being caught in strong H_2SO_4 (7 parts H_2SO_4 to 1 part

[1] A hydrated aluminum silicate obtained from Eli Lilly & Co., Indianapolis, Ind.

H_2O). The $(NH_4)_2SO_4$ in the acid was then returned to the flask and digestion effected as in the Kjeldahl-Gunning method, 5 grams of powdered K_2SO_4 being used to raise the temperature of the digesting liquid. Before distillation 25 ml. of 4 per cent K_2S solution were added to precipitate the mercury from mercurammonium compounds. Where the determination did not involve nitrates the Gunning modification of the Kjeldahl method as given on page 7 of 'Official Methods' (Association of Official Agricultural Chemists, '21) was used. Trouble from bumping during distillation was entirely overcome by the addition of approximately a tablespoonful of glass beads and a gram of powdered pumice stone to each flask. In my hands the official Gunning method modified to include the N of nitrates (page 8 of 'Official Methods') was entirely unsatisfactory, even with the utmost precautions; particularly if the "hypo" was added all at once a brownish gas could frequently be seen above the liquid, indicating a loss of N. This was to all appearances overcome by adding the thiosulphate slowly and shaking continuously during the addition, but the results on known solutions of nitrate were very variable. The following show the trend of the results obtained by the official method.

TABLE I

TOTAL NITROGEN INCLUDING NITRATES

	Ml. 1055 *N* H_2SO_4	Mgm. N	Theory
10 ml. of 0.1 *M* KNO_3 solution	9.15	13.530	14.010
5 ml. of 0.1 *M* KNO_3 solution	4.05	5.986	7.005
5 ml. of 0.1 *M* KNO_3 solution	4.15	6.134	7.005
10 ml. of 0.1 *M* NH_4NO_3 solution	17.60	26.010	28.020
5 ml. of 0.1 *M* NH_4NO_3 solution	9.30	13.750	14.010

The method outlined at the beginning of this paragraph gave dependable results and was much more rapidly carried out than the official method.

So far as I have been able to determine, there is no satisfactory method for the determination of nitrate N in the presence of ammonium and other forms of N and organic substances. Scales' ('16) method of reduction with Zn-Cu couple gives accurate results, if great precaution is taken with the couple. However,

the time and trouble required to clean and renew the material and in adding the NaCl and MgO, together with the necessity of running blanks without couple where the solution contained NH_4N, made it inadvisable to use this method. The difficulty of the proper execution of the old gasometric method of Schulze-Tiemann (Emmerling, '12) is recognized by those who have employed it. The method of Strowd ('20) was adopted because of its simplicity and its fair degree of accuracy. In this the nitrates are reduced to NH_3 by means of Devarda's alloy in the presence of alkali, blanks with alkali but no alloy being "run" at the same time under as nearly similar conditions as possible. This did not give dependable results, however, in the presence of dextrose, the sugar in some way holding back some of the nitrate. Accordingly the results obtained with media containing sugar, as given in the following data, are not considered reliable. The results obtained in the third series with *Aspergillus niger* are shown in the graphs (fig. 10), because they indicate relatively the course of nitrate consumption by this fungus; moreover, the sugar being entirely consumed by this organism in 5 days, the results for nitrate carry more weight. In subsequent series the nitrate was determined only after the disappearance of the sugar, and the results given were obtained by subtracting the result for NH_4 from that for total N.

Nitrites were determined in the first series with *Aspergillus niger*, a "micro" modification of the official method (page 22, 'Official Methods'), as applied to water analysis, being developed and used. In the official method the color ratios are obtained by varying, in 1 ml. quantities or multiples thereof, the amount of standard nitrite in the 50 or 100 ml. of H_2O, each ml. of standard containing 0.0001 mgm. of nitrite. The same quantities of reagents are added to each tube of standard and unknown. In the "micro" method the proportions of standard nitrite to 5 ml. water were maintained by use of a pipette that delivered 20 drops per ml. It is seen, therefore, that each drop of standard nitrite in 5 ml. of H_2O corresponds to the 1 ml. of standard nitrite in 100 ml. of H_2O. The standards were made in small, serological test-tubes marked at 5 ml. The same corresponding amounts of the reagents (concentrated HCl diluted 10 times,

sulphanilic acid, and a-naphthylamine-HCl) were then added to the standards and 5-ml. portions of unknowns. One or more drops of the respective reagents may be employed so long as the amounts are constant for each test. The same concentration of HCl as given in the official method is obtained by diluting the C.P. HCl to one-tenth its strength. The solutions are allowed to stand 30 minutes before the color comparisons are made. The standards are made up fresh each time determinations are made. Other comparable ratios for pipettes delivering other than 20 drops per ml. are readily calculated, and a volume other than 5 ml. may be chosen if the proper adjustments are made. The advantages of the "micro" method are the ease of making quickly a large number of standards, the economy in the use of reagents and unknown solution, and the more accurate comparison of the unknowns and standards by use of the comparator block and the Duboscq colorimeter. Turbidity and color is compensated for the same as in the hydrion determinations.

Amino nitrogen determinations were made on the material aerated in the NH_3 estimations by means of the van Slyke "micro" method in which 2 ml. of unknown solution were used. The aeration removes NH_3 and free amides which would interfere, giving abnormally large quantities of free N. Several preliminary experiments showed that an interfering NH_4 salt may be separated from an amino acid in this way. One of these experiments is here given. Two 10-ml. samples of 0.1 *N* solution of $(NH_4)_2SO_4$ were added respectively to two 10-ml. quantities of an approximately 0.1 *N* alanine solution, 1 gm. K_2CO_3 added, and the combined solutions aerated for separation and determination of the NH_3.N. Two 10-ml. samples of 0.1 *N* $(NH_4)_2SO_4$ plus 1 gm. K_2CO_3 were aerated alone, as were 2 similar quantities of alanine solution. Three 10-ml. portions of the $(NH_4)_2SO_4$ solutions were estimated by the Kjeldahl method, as were two 10-ml. aliquots of alanine.

TABLE II

EFFICIENCY OF AERATION METHOD FOR SEPARATION OF (NH_4) AND (NH_2)N

	Ml. 1055 *N* H_2SO_4	Mgm. N
1. N from $(NH_4)_2SO_4$ by Kjeldahlization	9.10 9.15 9.15	13.52 13.52
2. N from alanine by Kjeldahlization	9.10 9.10	13.538
3. N from $(NH_4)_2SO_4$ by aeration	9.10 9.10	13.45
4. N from $(NH_4)_2SO_4$-alanine by aeration	8.95 9.05	13.376
5. N from alanine by aeration	—	—
	Ml. N_2	Mgm. NH_3. N
6. Amino N from 0.05 N alanine, 2 ml.		
Before aeration 24° C.—766 mm.	2.33	1.3106
24° C.—766 mm.	2.35	1.3218
22° C.—766 mm.	2.35	1.3348
After aeration 22° C.—766 mm.	2.35 2.34	1.3348 1.329
7. Amino N from alanine $(NH_4)_2SO_4$		
After aeration 21° C.—766 mm.	2.338	1.335
21° C.—766 mm.	2.355	1.3447

These experiments show that aeration for 18 hours at the rate of 30–50 liters per hour is a satisfactory means of determining NH_3.N, and of separating NH_3 from amino N. Glucose and other constituents of the media were not found to interfere with the completeness of aeration.

The van Slyke "micro" method ('11, '11a, '12, '13, '15), rather than the "macro," was adopted because the apparatus used in the former is much more stable, requires only a fifth of the quantity of reagents, and the modification is as accurate as the original "macro" method. Special precautions must be observed in this determination. A definite period of reaction must be adopted and maintained throughout a given piece of work to obtain comparable results, because the blanks with water as well as the determinations on solutions containing NH_2.N vary with the time. In all the following work 5 minutes

were adopted as the time of reaction. A large number of determinations were made with distilled H_2O and various solutions to study the effect of duration of reaction on the quantity of N_2 evolved. A few determinations with 2 ml. of water are here given:

	Time in minutes	ml. N_2
Temperature 23° C.	3	.08
	5	.085
	5	.09
	8	.11
Barometer 763 mm.	20	.12
	40	.15

The blank appears to vary regularly with the quantity of gas given off by the nitrite. Variations in the blanks are to be expected with different supplies and grades of nitrite and acetic acid. Potassium nitrite cannot be used because the reaction of this nitrite with glacial CH_3COOH is too vigorous. Where caprylic alcohol is used to overcome foaming a new blank must be run. The speed of shaking should also be maintained as nearly constant as possible. The time required to rid the reaction vessel of air may be materially lessened by warming the nitrite to 30° C. and shaking by hand during this preliminary process. The motor, however, adjusted to a suitable and definitely maintained speed, should always be used for the reaction shaking. To obtain reliable results the above precautions and the following, as advised by Dr. D. W. Wilson, Department Physiological Chemistry, Johns Hopkins Medical School, should be carefully observed. The stopcocks, especially the one just above the gas burette, should be kept well greased with the vaseline-paraffin-rubber preparation recommended by van Slyke; this should be done after every 3 or 4 determinations. After 200–300 determinations the stopcocks should be ground gently with powdered emery and oil followed by talcum in water. The method is not very satisfactory for solutions containing peptones or proteins; accordingly the results given for "peptid" N of the two peptone media are to be more dependable than those for "total amino."

In the procedure for determining "peptid" N a 50-ml. portion of the medium was hydrolyzed 3 to 5 hours with 20 per cent HCl,

the "humin" N filtered off, and the filtrate and washings then neutralized with saturated NaOH solution and made to volume (100 ml.). A portion of this solution was made distinctly alkaline to phenolphthalein, aerated to rid it of amide and ammonium N, and the "peptid" N estimated by the van Slyke "micro" method, as in the amino N determinations. The results for amino N theoretically should be subtracted from the results obtained here to give the N that was bound in the peptid linkings and freed by hydrolysis, but this was not done owing to the questionable accuracy of the former. Hydrolysis of culture fluid from the 3 mineral N media (numbers 3, 4, and 5) was not found to increase the NH_2.N content, so it was reasoned that higher peptids, or proteins, are not excretion products of the 3 fungi cultured on these media.

From the NH_3 obtained by the aeration of the hydrolyzed material was subtracted that of the ammonia determination and the result called "amide" nitrogen.

Data and Discussion

A preliminary experiment was carried out to obtain an indication of the capacity or inability of *Sphaeropsis malorum* and *Diplodia natalensis* to utilize elementary N_2. These fungi are sphaeropsidaceous forms, as is the *Phoma Betae* which was shown to have the capacity for fixing free N_2. The Ascomycete, *Nectria Ipomoeae*, was also used in this experiment. The technique described by Duggar and Davis ('16) was followed exactly except that 1-liter, round-bottom Jena flasks were used as the culture, digestion, and distillation flasks. The cultures were incubated in darkness at a temperature of 28° C. Duggar's solution for fungi was used as the culture medium, the carbon source being common cane sugar instead of glucose, and the N source 2 different concentrations of peptone solution. The results here given show the total incapacity of these 3 forms to fix the N_2 of the air under the conditions of the experiment.

TABLE III

NITROGEN FIXATION

Organism	Days incubation	Mgm. N in culture	Mgm. N in check
Nectria Ipomoeae	14	34.82	
	14	35.11	
	14	34.77	34.82
	14	35.39	34.76
	14	35.05	
Nectria Ipomoeae	14	18.09	17.98
Nectria Ipomoeae	29	34.94	
	29	34.82	34.82
Sphaeropsis malorum	14	34.26	
	14	35.56	34.71
	14	34.94	34.71
Sphaeropsis malorum	14	17.98	
	14	18.09	17.98
	14	18.38	18.03
Sphaeropsis malorum	29	34.65	
	29	34.71	34.71
Diplodia natalensis	14	36.19	
	14	35.51	35.79
Diplodia natalensis	14	18.72	
	14	18.78	18.09
	14	19.00	17.93
Diplodia natalensis	29	35.34	
	29	35.79	35.79

The analytical data for the work with *Aspergillus niger*, *Sphaeropsis malorum*, and *Diplodia natalensis* are given in the following tabulations and graphs. The heavy vertical lines of the graphs represent the days on which determinations were made. The $[\overset{+}{H}]$ of the "peptone plus dextrose" medium is seen to increase during the first 3 to 5 days in cultures of all 3 fungi; a comparison of the curves for dry weight and $[\overset{+}{H}]$ shows that the rise in $[\overset{+}{H}]$ is proportional to the rapidity and amount of growth of the fungi. For the very fast-growing *Aspergillus* the maxima of growth of the organism and $[\overset{+}{H}]$ of the medium are coincident in time; whereas for the other 2 fungi there is a distinct lag of 2 to 3 days in the growth maximum. That both these factors are intimately related to the carbohydrate consumption is

evident. With the disappearance of the dextrose in *Aspergillus* cultures a decrease in the weight of the fungus and in [$\dot{H}$] immediately begins. For the other 2 fungi the maximum dry

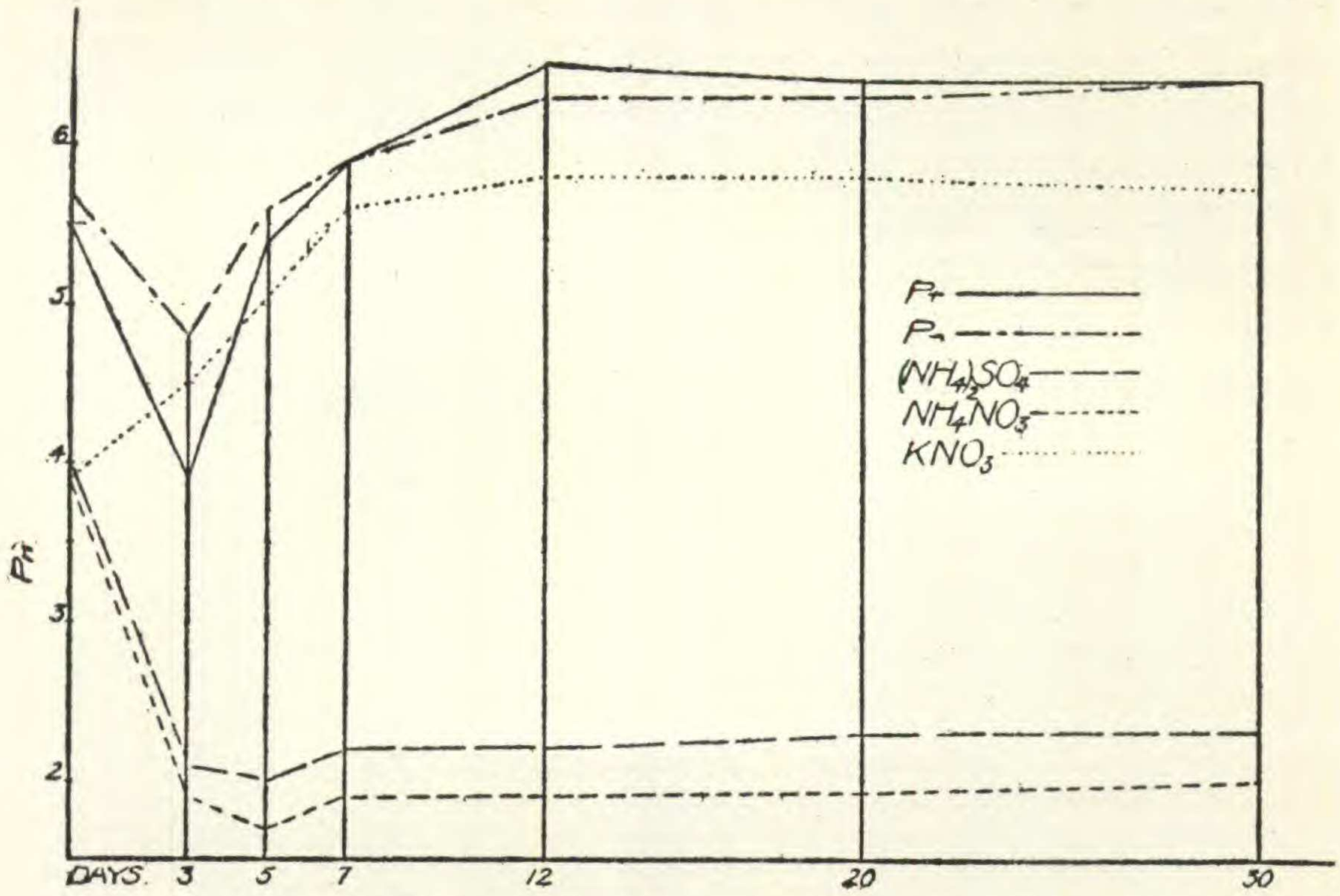


Fig. 1. H-ion change of media. *Aspergillus niger*, first series.

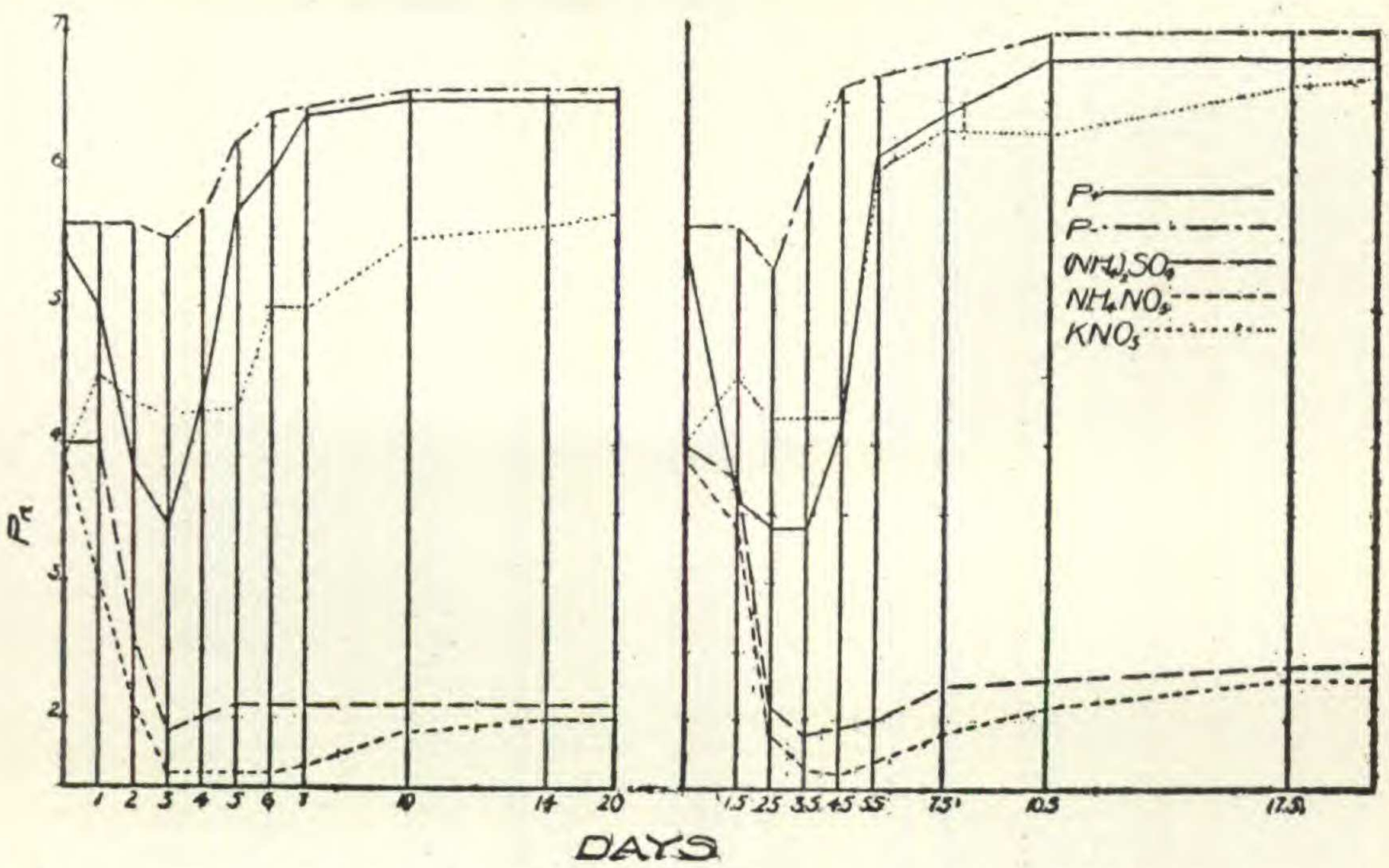


Fig. 2. H-ion change of media. *Aspergillus niger*, second (left) and third series.

weight and the point of disappearance of the sugar are synchronous. The organic acids produced in the decomposition of the glucose are probably responsible for the rapid elevation of H-ion concentration. The carbohydrate having been entirely transformed, the organic acids are rapidly built up into the substance of the fungus and consequently there is a rapid decrease in $[\overset{+}{H}]$, as indicated by the rapidly ascending curve for the $[\overset{+}{H}]$ exponent, P_H. This would explain the growth lag evidenced by the *Sphaeropsis* and *Diplodia*. This would probably have been found to be the case also with the faster-growing *Aspergillus*, had determinations been made at intervals of 5 or 6 hours rather than days.

TABLE IV

CHANGES IN H-ION CONCENTRATION, DEXTROSE, TOTAL N, AND NITRITE N OF THE MEDIA, AND IN DRY WEIGHT OF FUNGOUS MAT

ASPERGILLUS NIGER—FIRST SERIES

All weights expressed in mgm. per 50 ml. of media

Medium	Days incubation	Number of cultures	P_H	Dry wt. Mat No. 1	Dry wt. Mat No. 2	Dry wt. Mat No. 3	Average dry wt. mats	Total N	NO_2. N	Dextrose
	0	5	5.5					228.4	0	2477
Peptone	3	5	3.9	1837	1810	1713	1787	120.0	.0012	Trace
plus	5	5	5.4	1321	1290	1336	1316	125.2	.0010	0
dextrose	7	5	5.9	1172	1192	1030	1132	149.5	0	
	12	5	6.5	1116	1078	1129	1108	154.7	.0125	
No. 1	20	5	6.4	1116	1120	1144	1127	139.1	.0073	
	30	5	6.4	1069	1070	1045	1061	146.1	.0025	
Peptone	0	5	5.7					214.5	0	0
minus	3	5	4.8	222	271	232	242	189.2	.0005	
dextrose	5	5	5.6	338	263	312	304	208.6	.0007	
	7	5	5.9	294	258	258	273	208.6	.0087	
No. 2	12	5	6.3	249	237	238	241	187.8	.0062	
	20	5	6.3	250	252	227	243	196.5	0	
	30	5	6.4	224	255	177	219	201.7	.0020	
	0	5	4.0					284.0	0	2477
	3	5	2.1	1009	981	923	971	246.9	.0005	365
$(NH_4)_2SO_4$	5	5	2.0	1060	982	1149	1063	236.4	.0002	0
	7	5	2.2	907	830	937	891	239.9	.0001	
No. 3	12	5	2.2	703	682	740	708	246.9	0	
	20	5	2.3	856	634	682	724	245.1	0	
	30	5	2.3	644	677	742	688	255.6	0	

TABLE IV (*Continued*)

Medium	Days incubation	Number of cultures	P_H	Dry wt. Mat No. 1	Dry wt. Mat No. 2	Dry wt. Mat No. 3	Average dry wt. mats	Total N	NO_2. N	Dextrose
	0	5	3.9					280.0	.0010	2477
	3	5	1.9	1161	1116	1199	1159	219.1	.0005	Trace
NH_4NO_3	5	5	1.7	1017	1044	1044	1035	205.2	.0005	0
	7	5	1.9	927	885	913	908	227.8	0	
No. 4	12	5	1.9	818	864	865	849	246.9	0	
	20	5	1.9	775	765	877	806	239.9	0	
	30	5	2.0	766	722	747	745	248.0	0	
	0	5	3.9					149.5	.0005	2477
	3	5	4.5	821	993	888	901	97.4	.0025	787
KNO_3	5	5	5.5	1031	1000	1189	1073	90.4	.0067	0
	7	5	5.6	791	820	889	830	95.6	.0162	
No. 5	12	5	5.8	740	778	732	750	90.4	.0085	
	20	5	5.8	786	702	737	742	104.3	.0010	
	30	5	5.7	804	633	738	725	111.3	0	

TABLE V

CHANGES IN H-ION CONCENTRATION, DEXTROSE, AND NITROGEN CONSTITUENTS OF MEDIA, AND IN DRY WEIGHT OF FUNGOUS MAT

ASPERGILLUS NIGER—SECOND SERIES

All weights expressed in mgm. per 50 ml. of media

Medium	Days incubation	Number of cultures	P_H	Average dry wt. fungous mat	Dextrose	NH_4.+NH_3.N	Total N	NO_3.N	NH_3.N	$CONH_2$.N	CONH.N
	0	5	5.4	0	2255	1.38	176.7	0	27.23	4.14	76.51
	1	1	5.0	113	2212						
	2	1	3.8	615	1235						
Peptone plus dextrose	3	5	3.4	1445	47	1.38	86.94		7.56	4.14	46.7
	4	1	4.3	1279	18						
	5	5	5.7	1032	18	30.36	102.8		10.1	2.76	37.64
No. 1	6	1	6.0	1110							
	7	5	6.4	1034		33.81	91.77		10.38	3.45	30.44
	10	5	6.5	1004		46.92	113.2		12.73	1.1	37.23
	14	5	6.5	985		48.3	108.3		11.72	0	32.72
	20	5	6.5	946		51.06	106.9		12.88	12.4	28.51
	0	5	5.6	0	0	2.76	176.7	0	31.6	2.76	93.58
	1	1	5.6	60							
	2	1	5.6	225							
Peptone minus dextrose	3	5	5.5	157		35.88	153.2		11.6	13.8	54.8
	4	1	5.7	215							
	5	5	6.2	168		64.86	160.1		16.05	6.9	49.36
	6	1	6.4	233							
No. 2	7	5	6.4	199		68.31	152.5		11.21	2.07	44.83
	10	5	6.6—	187		77.28	162.8		16.1	0	43.25
	14	5	6.6—	178		80.04	162.1		13.08	2.76	41.71
	20	5	6.6	156		78.66	160.1		14.25	13.8	49.07

TABLE V (*Continued*)

Medium	Days incubation	Number of cultures	Pʜ	Average dry wt. fungous mat	Dextrose	$NH_4.+NH_3.N$	Total N	$NO_3.N$	$NH_2.N$	$CONH_2.N$	CONH.N
	0	5	4.0	0	2255	274.0	277.7	0	0	0	0
	1	1	4.0	85	2244						
	2	1	2.6	387	1525						
$(NH_4)_2SO_4$	3	5	1.9	1115	88	205.6	211.2		0		
	4	1	2.0	1045	3						
No. 3	5	5	2.1	822		229.1	227.0		.27		
	6	1	2.1	882							
	7	5	2.1	866		193.2	197.3		.97		
	10	5	2.3	816		209.7	222.2		1.52		
	14	5	2.3	761		219.4	231.2		4.9		
	20	5	2.3	754		229.7	233.2		1.23		
	0	5	3.9	0	2255	143.1	285.7	142.6	1.1	0	0
	1	1	4.0	69	2225						
	2	1	2.1	425	1674						
NH_4NO_3	3	5	1.6	866	185	96.6	221.5	124.9	0		
	4	1	1.6	1039	7						
No. 4	5	5	1.6	918		95.2	233.6	138.4	.27		
	6	1	1.6	1006							
	7	5	1.7—	899		87.3	224.3	137.0	7.26		
	10	5	1.9+	733		116.3	249.8	133.5	2.21		
	14	5	2.0+	697		118.7	260.8	142.1	1.36		
	20	5	2.0+	639		117.3	258.7	141.4	1.1		
	0	5	3.9	0	2255	1.38	142.8	141.4	0	0	0
	1	1	4.5	98	2243						
	2	1	4.3	345	1770						
KNO_3	3	5	4.2	413	1082	1.38	104.9	103.5	0		
	4	1	4.0	756	581						
No. 5	5	5	4.0	833	72	1.38	95.2	93.8	.54		
	6	1	5.0	786	5						
	7	5	5.0	792	4	2.07	95.2	93.1	.55		
	10	5	5.5	657		4.14	110.4	106.3	.83		
	14	5	5.6	637		2.76	112.5	109.7	2.04		
	20	5	5.7	667		4.83	107.7	102.8	.55		

TABLE VI

CHANGES IN H-ION CONCENTRATION, DEXTROSE, AND NITROGEN CONSTITUENTS OF MEDIA, AND IN DRY WEIGHT OF FUNGOUS MAT

ASPERGILLUS NIGER—THIRD SERIES

All weights expressed in mgm. per 50 ml. of media

Medium	Days incubation	Number of cultures	P_H	Average dry wt. mats (mgm.)	Dextrose	$NH_4.+NH_2.N$	Total N	$NO_3.N$	$NH_2.N$	$CONH_2.N$	CONH.N
	0	5	5.4	0	2540	1.38	176.7	0	27.52	4.14	76.51
	1½	1	3.6	186	1920						
Peptone	2½	1	3.4	738	982						
plus	3½	5	3.4	1512	55.6	4.14	93.84		10.8	0	43.1
dextrose	4½	1	4.1	1549	5.2						
	5½	5	6.1	1235	.0	22.1	82.8		8.7	1.38	34.9
No. 1	7½	5	6.4	1168		26.2	82.8		7.01	4.14	26.6
	10½	5	6.8	928		37.2	96.6		9.24	2.82	24.6
	17½	5	6.8	852		42.1	93.32		8.9	2.74	26.9
	27	5	6.8	865							
	0	5	5.6	0	0	2.7	176.7	0	32.7	2.76	93.6
	1½	1	5.6	73							
Peptone	2½	1	5.3	225							
minus	3½	5	6.0	212		37.3	152.5		14.8	0	61.3
dextrose	4½	1	6.6	192							
	5½	5	6.7	218		55.2	152.0		16.2	5.52	59.9
No. 2	7½	5	6.8	214		56.6	151.8		11.2	2.76	43.6
	10½	5	7.0	183		63.5	151.8		10.6	1.38	36.0
	17½	5	7.0	198		69.0	153.2		14.1	0	39.8
	27	5	7.0	199							
	0	5	4.0	0	2540	274.	277.7		0	0	0
	1½	1	3.5	160	2280						
$(NH_4)_2SO_4$	2½	1	2.1	580	1500						
	3½	5	1.9	1126	213	212.5	216.6		2.27		
No. 3	4½	1	2.0—	1144	0						
	5½	5	2.0	1032		213.9	234.6		2.8		
	7½	5	2.3—	767		200.1	205.6		1.68		
	10½	5	2.3	744		204.2	209.7		1.96		
	17½	5	2.4	684		200.1	204.2		2.25		
	27	5	2.4	646							
	0	5	3.9	0	2540	142.2	285.6	139.4	0	0	0
	1½	1	3.4	86	2310						
NH_4NO_3	2½	1	1.9	510	1533						
	3½	5	1.7—	897	86.4	96.6	220.8	123.9	.43		
No. 4	4½	1	1.6	965	2.9						
	5½	5	1.7	919		96.6	226.3	125.6	1.96		
	7½	5	1.9	796		103.5	234.6	128.3	1.68		
	10½	5	2.1	680		113.1	247.	128.3	.84		
	17½	5	2.3	616		113.1	245.7	127.0	1.41		
	27	5	2.3	616							

TABLE VI (*Continued*)

Medium	Days incubation	Number of cultures	P_H	Average dry wt. mats	Dextrose	$NH_4+NH_3.N$	Total N	$NO_3.N$	$NH_3.N$	$CONH_2.N$	CONH.N
	0	5	3.9	0	2540	1.3	142.8	138.0	0	0	0
	1½	1	4.5	90	2390	trace					
KNO,	2½	1	4.2	410	1705						
	3½	5	4.2	607	1507		107.6	102.1			
No. 5	4½	1	4.2	736	242						
	5½	5	6.0	868	18	1.38	95.2	86.94	1.82		
	7½	5	6.3	649		5.52	104.9	96.6	2.8		
	10½	5	6.2+	602		4.14	115.9	97.98	.56		
	17½	5	6.6	612		9.66	109.2	95.22	1.13		
	27	5	6.8	525							

TABLE VII

CHANGES IN H-ION CONCENTRATION, DEXTROSE, AND NITROGEN CONSTITUENTS OF MEDIA, AND IN DRY WEIGHT OF FUNGOUS MAT

SPHAEROPSIS MALORUM

All weights expressed in mgm. per 50 ml. of media

Medium	Days incubation	Number of cultures	P_H	Average dry wt. fungous mat	Dextrose	$NH_4.+NH_4.N$	Total N	$NO_3.N$	$NH_3.N$	$CONH_2.N$	CONH.N
	0	5	5.5	0	2452	1.5	154.8	0	33.2	7.6	90.1
	2	5	5.4	14	2414	1.6	150.2				
Peptone	3	5	5.0	124	2048	1.6	143.4				
plus	4	5	4.5	382	1452	1.6	129.7				
dextrose	5	1	4.0	884	415		96.7				
	6	5	4.4	1078	68	1.8	72.8				
No. 1	7	2	5.2	1293	16	3.4	68.3		16.7	3.4	39.2
	12	5	7.8	1114		31.8	79.7				
	16	2	8.1	914		44.4	88.7		13.4	1.12	25.66
	35	1	7.8	594		48.9	109.3		18.7		
	0	5	5.7	0	0	1.5	154.8	0	36.8	2.14	95.7
	2	5	5.8	4		1.8	153.6				
Peptone	3	5	5.9	15		3.18	154.8				
minus	4	5	6.2	52		7.96	152.5				
dextrose	6	5	6.6	159		18.2	136.6				
	7	2	6.9	192		23.9	129.7		21.1	5.7	61.12
No. 2	12	5	7.6	215		37.55	132.0				
	16	5	8.1	185		44.3	132.0		27.1	.7	48.75
	35	1	7.8	115		45.5	130.9		23.2		

TABLE VII (*Continued*)

Medium	Days incubation	Number of cultures	Ph	Average dry wt. fungous mat	Dextrose	$NH_4.+NH_3.N$	Total N	$NO_3.N$	$NH_2.N$	$CONH_2.N$	CONH.N
	0	5	4.2	0	2452	245.8	247.0	0	1.4	0	0
	2	1	4.1	2	2439	245.8	247.				
	3	1	4.0	12	2428	245.3	247.				
$(NH_4)_2SO_4$	6	5	3.6	39	2247	243.9	245.3				
	8	5	3.3	98	1671	239.4	242.4		2.25		
No. 3	9	2	2.8	146	1210						
	12	5	2.8	160	886	232.1	235.6				
	15	1	2.7								
	17	2	2.7	193	680	228.7	233.9		3.9		
	0	5	4.0	0	2452	124.4	250.3	125.9	.56	0	0
	2	5	4.0	3	2452	124.4	246.9	122.5			
	3	5	4.0—	3	2452	124.4	246.9	122.5			
NH_4NO_3	6	5	3.8	67	2274	122.9	246.9	122.5			
	8	5	3.8+	207	1651	118.8	237.8	119.0	.7		
No. 4	9	2	3.8+	363	1090						
	10	1	4.4	365	836						
	12	5	5.0	374	270	116.0	223.0	107.0			
	15	1	5.8	655	16						
	16	5	5.8	574		112.7	217.3	104.6	.57		
	0	5	4.0	0	2452	0	121.8	121.8	0	0	0
	2	5	4.0	5	2450	0	119.5	119.5			
	3	5	4.0	4	2450	0	119.5	119.5			
KNO_3	6	5	4.9	95	2112	0	119.5	119.5			
	8	5	5.7	377	1419	0	102.4	102.4	.14		
No. 5	9	2	5.8	523	779	0					
	10	1	5.8	733	176	0					
	12	5	6.0	761	69	0	88.7	88.7			
	16	2	6.6	715	12	0	88.7	88.7	.14		
	35	2	8.1	376		5.7	102.4	96.7	.14		

TABLE VIII

CHANGES IN H-ION CONCENTRATION, DEXTROSE, AND NITROGEN CONSTITUENTS OF MEDIA, AND IN DRY WEIGHT OF FUNGOUS MAT

DIPLODIA NATALENSIS

All weights expressed in mgm. per 50 ml. of media

Medium	Days incubation	Number of cultures	P_H	Average dry wt. fungous mat	Dextrose	$NH_4+NH_3.N$	Total N	$NO_3.N$	$NH_2.N$	$CONH_2.N$	$CONH.N$
	0	5	5.4	0	2367	0	154.77	0	33.22	9.1	90.1
	2	1	5.0	85	2276						
	3	5	4.7	279	1842	0	128.6				
Peptone	4	2	4.6	668	789	0					
plus	5	5	5.0	821	194	4.5	94.45				
dextrose	6	2	6.5	925	73	17.1					
No. 1	8	5	6.9	849	7 da. 63	22.8	91.04		19.7	4.55	46.23
	12	5	8.1	703		40.97	100.14				
	18	5	8.2	615		42.11	97.9		13.54	3.41	35.17
	38	2	8.2	597		42.11	91.04		11.15		28.02
	0	5	5.7	0		0	154.77	0	36.87	2.45	95.7
	3	5	5.8	15		3.	153.6				
	4	2	6.3	86							
Peptone	5	5	6.6	133		25.	144.5				
minus	6	2	7.0	185		25.					
dextrose	8	5	7.5	248		35.3	132.0		23.74	3.4	56.72
No. 2	11	1	8.3	259							
	12	5	8.4	251		39.8	128.6				
	18	5	8.5	223		44.38	119.5		19.12	5.69	42.22
	38	2	8.4	203		43.24	116.1		17.44		31.45
	0	5	4.2	0	2367	245.8	246.9	0	1.4	0	0
	3	5	3.9	28	2360	243.5	243.5				
	4	2	3.4	78	2180						
	5	5	2.8	124	1606	233.3	236.7				
$(NH_4)_2SO_4$	6	2	2.7	203	1006	225.3					
No. 3	8	5	2.6	186	718	220.7	234.4		4.5		
	10	1	2.6	193	511						
	12	5	2.6	217	509	223.	232.1				
	18	5	2.6	232	428	220.	229.8		6.0		
	38	2	2.6	355	41	208.3	220.8		6.0		
	0	5	4.0	0	2367	124.1	250.4	126.3	.56	0	0
	3	5	3.9	23	2360	124.	245.8	121.8			
	4	2	3.8	45	2297						
	5	5	3.4	133	1845	119.5	240.1	120.6			
NH_4NO_3	6	2	3.2	187	1492	118.35					
No. 4	8	5	3.3	387	67	104.7	219.6	114.9	1.7		
	9	1	3.6	415	21						
	12	5	2.9	474		102.4	213.9	111.5			
	18	5	2.7	368		101.3	221.9	120.6	3.9		
	38	2	2.7	391		101.3	216.2	114.9	4.0		

TABLE VIII (*Continued*)

Medium	Days incubation	Number of cultures	P_H	Average dry wt. fungous mat (mgm.)	Dextrose	$NH_4.+NH_3.N$	Total N	$NO_3.N$	$NH_2.N$	$CONH_2.N$	CONH.N
	0	5	4.0	0	2367	0	121.8	121.8	0	0	0
	3	5	4.1	30	2295	0	111.5	111.5			
	4	2	4.8	53	2230						
	5	5	5.5	271	1654	0	104.7	104.7			
KNO_3	6	2	5.7	326	1554	0					
No. 5	8	5	5.8	632	413	0	87.63	87.63	1.13		
	9	1	7.0	759	28						
	12	5	7.1	613		2.2	87.6	85.4			
	18	5	7.8	485		7.97	96.73	88.76	1.26		
	38	1	7.8	422		7.97	89.9	81.93	1.28		

At this point in the growth of the fungi, since the carbohydrate carbon supply has been exhausted, the organisms must draw upon the peptone and probably upon their own substance for both C and N to continue their metabolic processes, especially

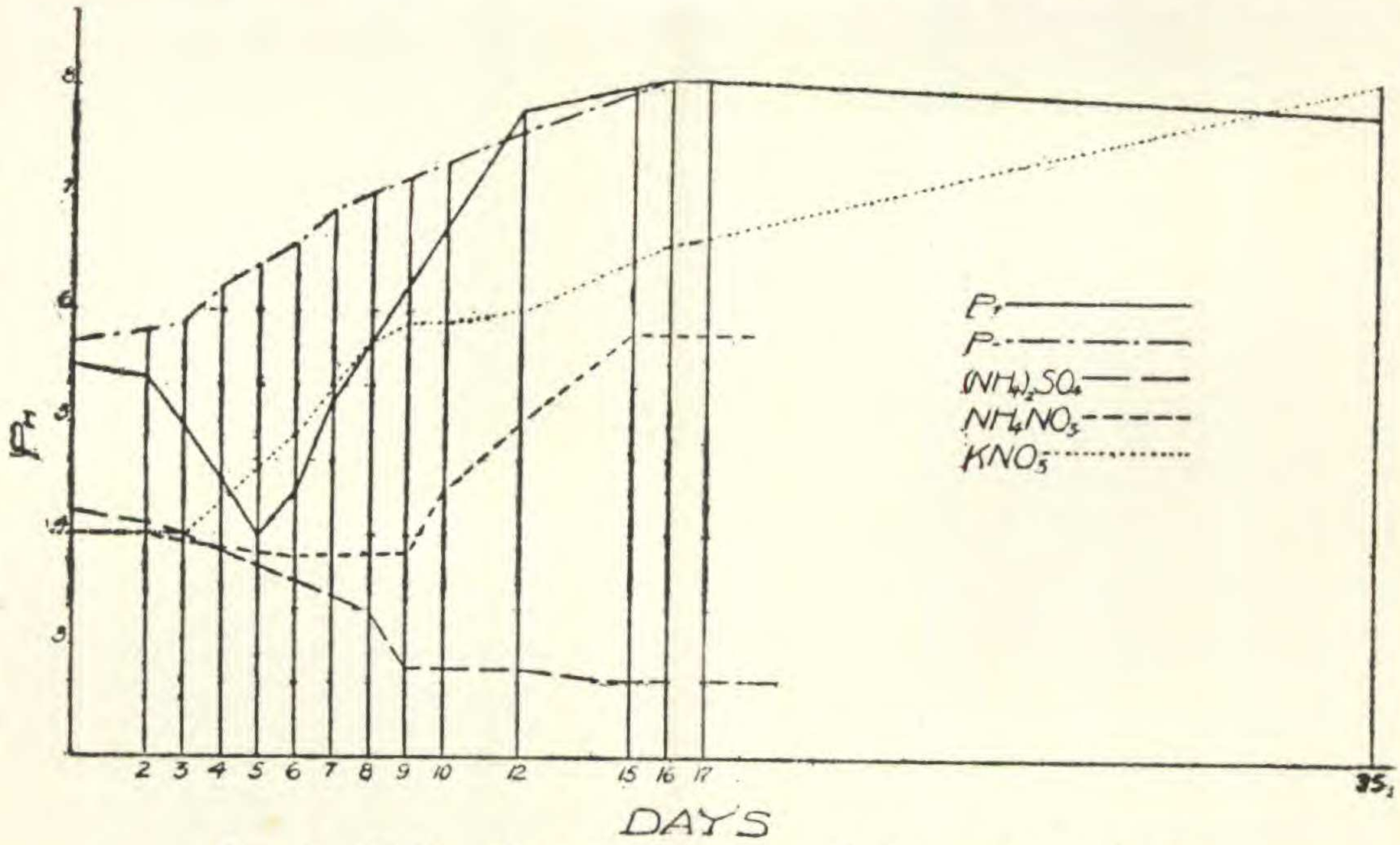


Fig. 3. H-ion change of media. *Sphaeropsis malorum.*

respiration. With the disappearance of the sugar there is a relatively rapid increase in the $NH_4.N$ of the medium. At least 2 factors may enter into an explanation of this. The proportion of N to the non-nitrogenous complex of the peptone is greater

than is required in respiration, protein synthesis, and other life processes; accordingly the excess N appears as NH_3. In the case of *Aspergillus niger* this rapid excretion of NH_3 in the "peptone minus dextrose" medium comes about 2 days before that in the "peptone plus dextrose" medium, and respectively about 3 and 5 days earlier for cultures of *Diplodia* and *Sphaeropsis*, showing the protective action of the sugar. Decrease in weight of the fungus indicates autolysis and the consequent diffusion of autolytic products into the medium. Ammonia is very likely one of the products of this autolysis. The results for

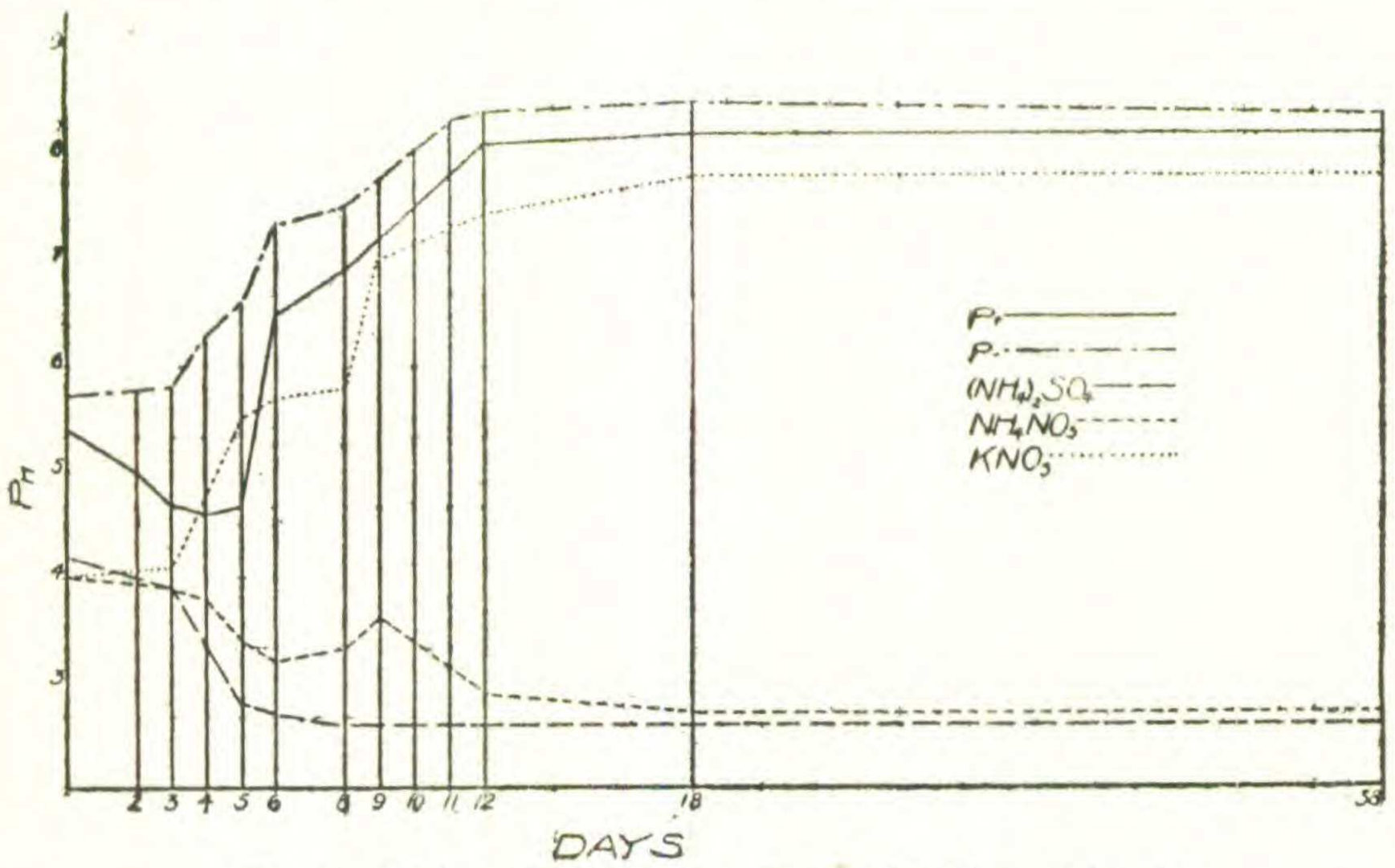


Fig. 4. H-ion change of media. *Diplodia natalensis*.

total N are also strikingly suggestive in this regard. With the decrease in weight of the fungi the N content of the media increases above its minimum, indicating that the autolytic products are in part nitrogenous. The results for amino, amide, and peptid N are not suggestive in this regard; in fact the peptid N of the medium in general decreased in quantity during the incubation of all 3 fungi. There is, however, a slight increase in the peptid N content of the medium of the *Aspergillus* cultures at the end of the incubation. These results indicate that NH_3.N is the chief nitrogenous product of autolysis. The quantity of NH_3 excreted reaches nearly a third of the total N of the medium in 18 to 20

days. The determinations, moreover, corroborate those of Dox and Maynard ('12 and '13).

While it is not known that NH_3 is an end product of protein metabolism, it is certain that it is directly related to autolytic processes; and in cultures where peptone or protein is the sole source of C and N, NH_3 is produced in excess by the breaking down of large numbers of molecules and the use of the non-N-containing parts for energy. In the experiment on N fixation

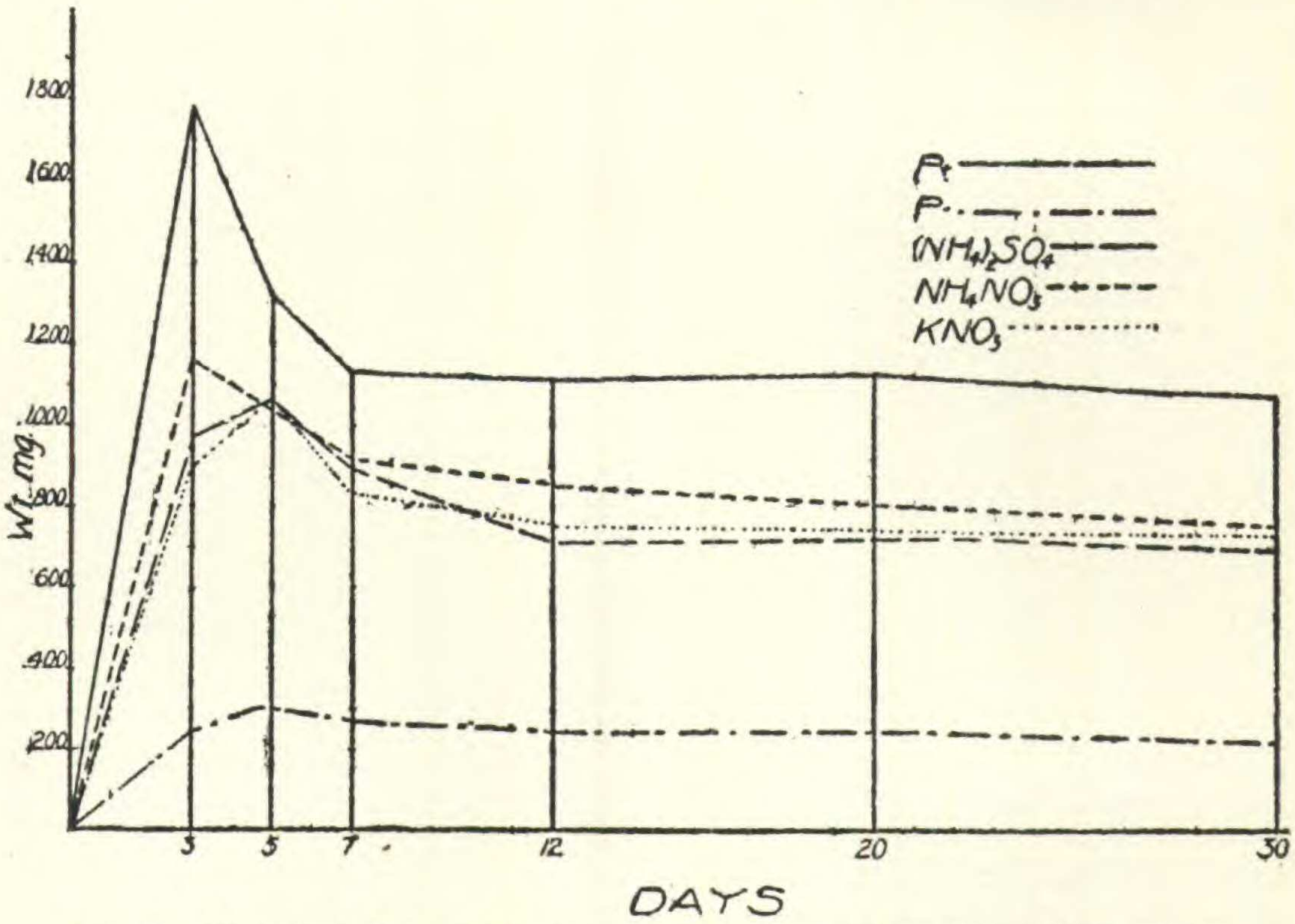


Fig. 5. Dry weights of fungous mats. *Aspergillus niger*, first series.

reported at the beginning of this discussion, it is significant that the N content of the cultures remained constant for 29 days. The initial N content of the media was very small, and any ammonia formed from the peptone in the presence of the abundant sugar was immediately reassimilated and retained. There was consequently no loss of N during 29 days of incubation. On the other hand, in the cultures of *Sphaeropsis* and *Diplodia* where the peptone content was large and the sugar disappeared in 7 to 8 days, there was a distinct loss of nitrogen when the cultures became alkaline. The strong odor indicated that the loss was due to the evolution of free NH_3. At the end of 15 days a culture

of *Sphaeropsis malorum* on the P medium was found, by Kjeldahlization of the mat and solution collectively, to have lost 6.77 mgm. of N; and one on P medium, 3.42 mgm. Similarly a 20-day P+ culture of *Diplodia natalensis* lost 7.97 mgm. N, and a 20-day P— culture, 9.106 mgm. On the 3 media in which inorganic N served as the N source none of the cultures decreased in total N content. The continued acidity of the $(NH_4)_2SO_4$ and NH_4NO_3 media prevents the evolution of NH_3, while the small amount of NH_3 produced in alkaline KNO_3 cultures is

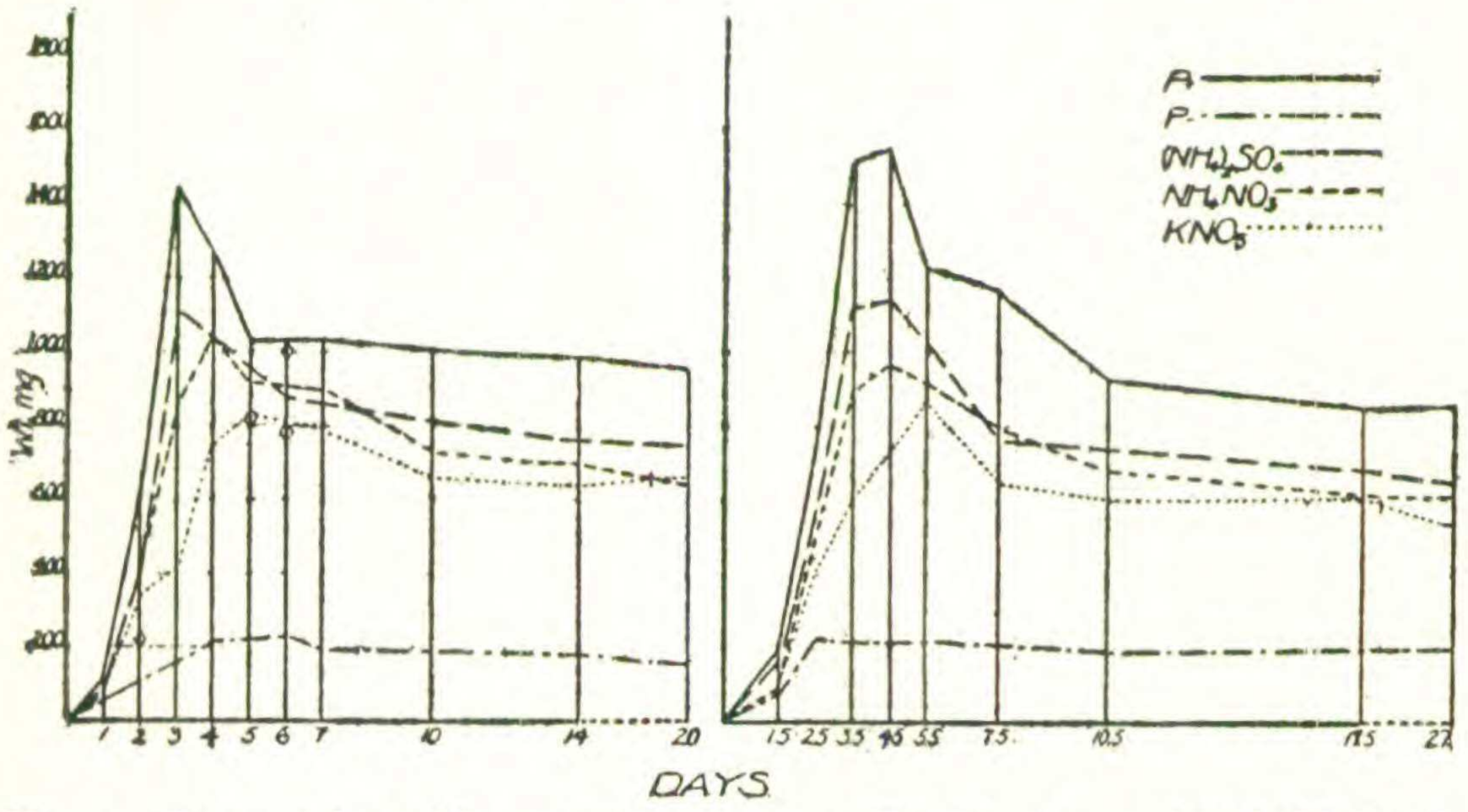


Fig. 6. Dry weights of fungous mats. *Aspergillus niger*, second and third series.

precipitated as $MgNH_4PO_4.6H_2O$ or easily held in solution as NH_4OH. This strengthens the probability that the NH_3 evolved under the alkaline conditions of the P+ and P— media is responsible for the N loss in these cultures.

Waksman ('18) did not make sugar- and dry-weight determinations and consequently could not legitimately connect the appearance of ammonia with autolysis and the disappearance of sugar. The sugar of his cultures had probably disappeared at the end of 3 to 5 days and consequently the obedience of the accumulation of NH_3 to the law of autocatalysis could not have been due to the presence of the carbohydrate, as he claimed. He is probably correct in assuming that a definite quantity of NH_3 may always be produced from protein materials, as a waste product, independently of the presence of available carbohydrate.

My results show that this ammonia is, as Waksman assumes, "reassimilated in the presence of available carbohydrate by the organisms that are able to utilize it readily as a source of N." In the case of *Sphaeropsis* and *Aspergillus* "the production of NH_3 was entirely prevented by the presence of the sugar." The relation also holds with *Diplodia*. but in cultures of this fungus an appreciable quantity of NH_3 (17.1 mgm. per culture) appeared when 73 mgm. of glucose were still present. This may have

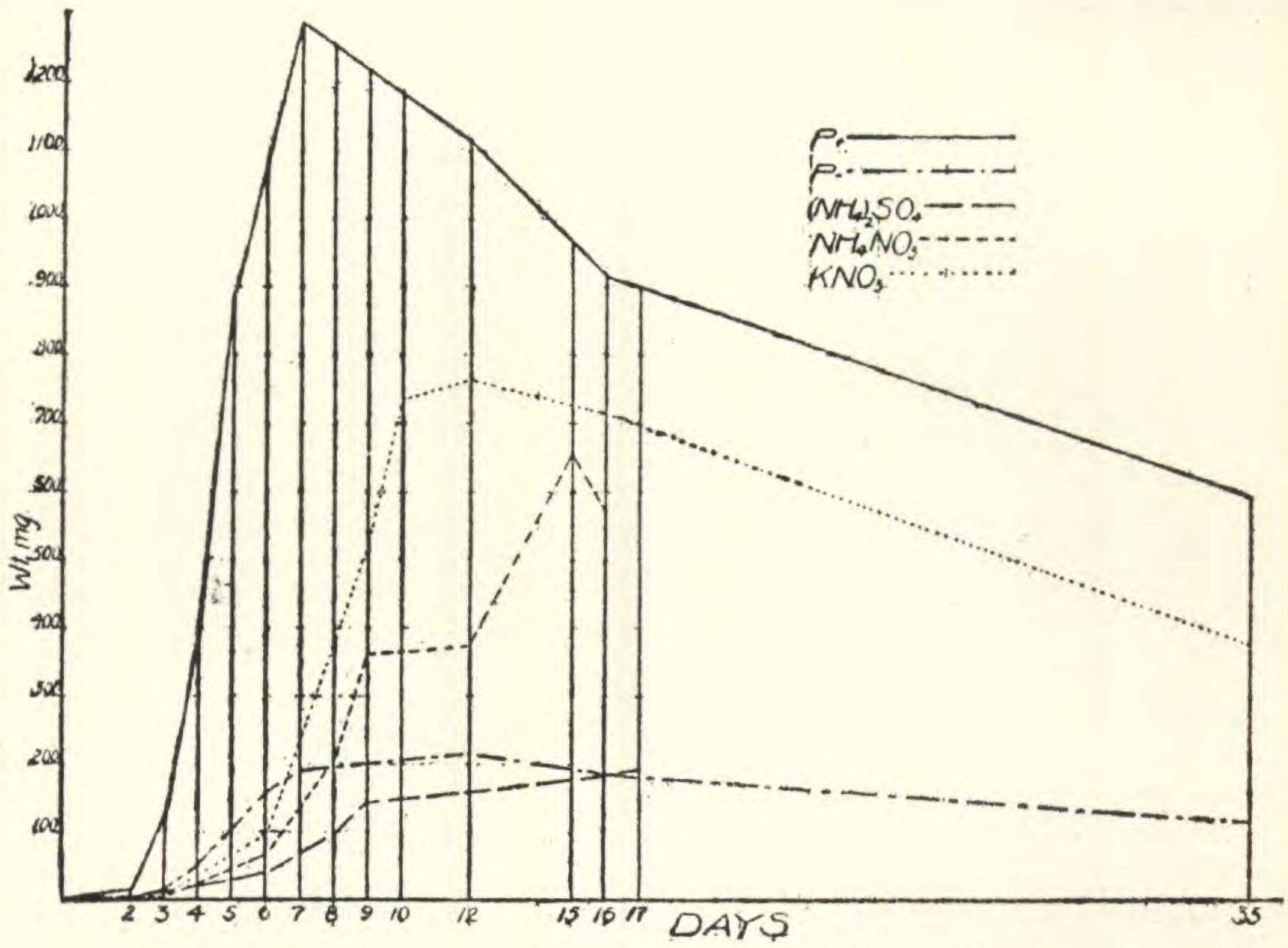


Fig. 7. Dry weights of fungous mats. *Sphaeropsis malorum*.

been due to the fact that the aerial, folded growth of the fungus minimized contact with the medium and thereby lessened absorption from it. In the absence of the dextrose, the strongly proteolytic organisms use the peptone as a source of C and leave much of the N to diffuse into the medium as NH_3.

As the alkalinity advanced beyond P_H 7.0 crystals formed in all the peptone media for all 3 fungi and in the KNO_3 medium of *Sphaeropsis* and *Diplodia* cultures. In the peptone cultures a strong odor of NH_3 was apparent, and crystals of $MgNH_4PO_4 \cdot 6H_2O$ were precipitated; in the KNO_3 medium with the *Diplodia*

and *Sphaeropsis* the precipitate was largely $Mg_3(PO_4)_2.4H_2O$, as the NH_3 content of these cultures does not reach an appreciable quantity until late in the incubation, when most of the Mg and PO_4 ions have been precipitated. The appearance of the characteristic crystals of the salts may be considered as a rough indication of the beginning of an alkaline reaction which, for the most part, results from predominance of autolytic processes. The reduction in active acidity necessary to their formation was determined experimentally by adding NH_4OH or KOH to the media; the

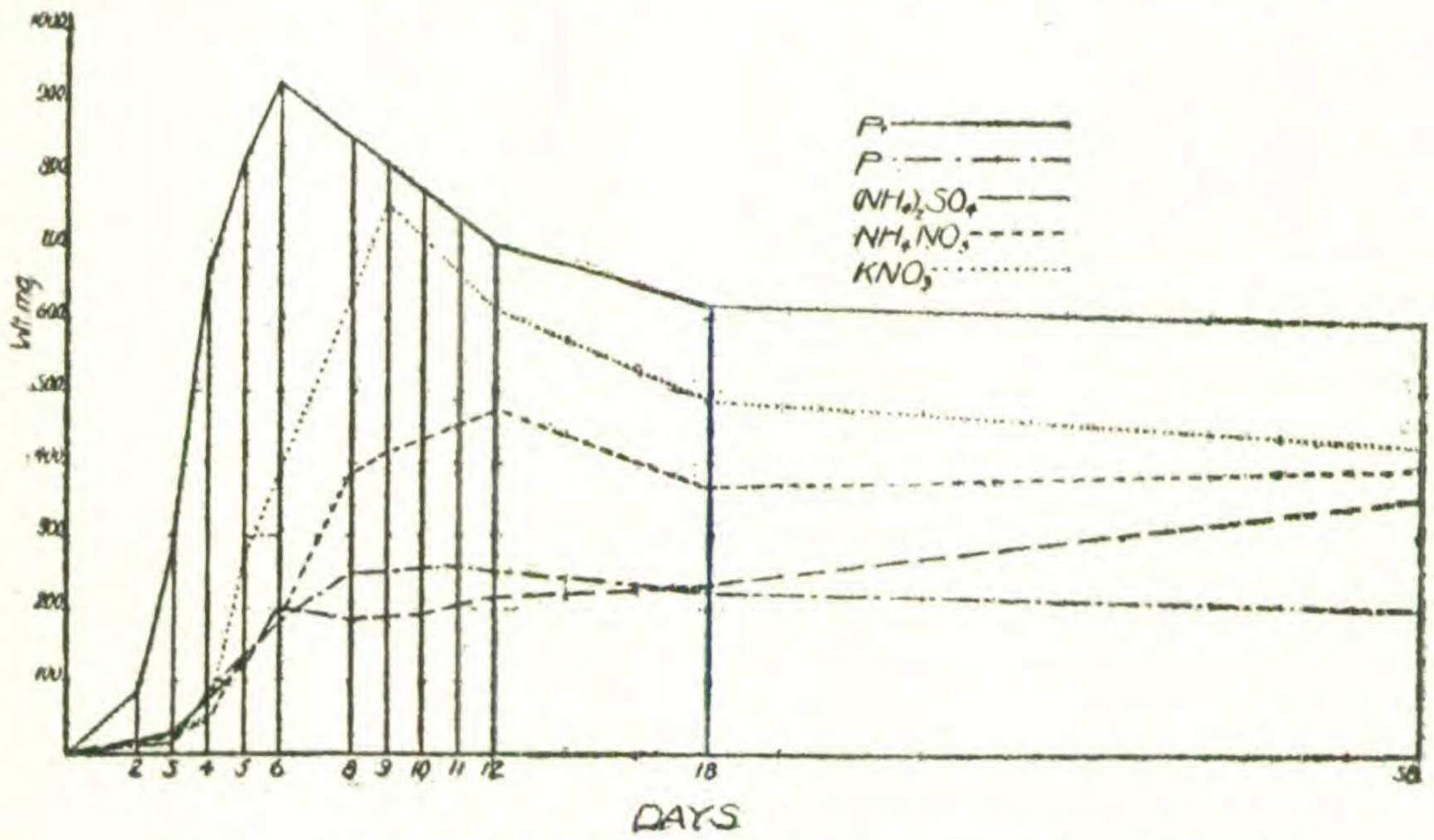


Fig. 8. Dry weights of fungous mats. *Diplodia natalensis*.

crystals were centrifuged out and identified by microscopic comparison with known salts and by analyzing quantitatively for N content and qualitatively for the ions present. Crystals appeared at P_H 6.1 in the presence of NH_3; that is, when NH_4OH was added to the media, or KOH to media 3 and 4 which contained NH_4 salts, these were identified as $MgNH_4PO_4.6H_2O$. When KOH was added to media 1, 2, and 5, crystals were not formed until the exponent P_H 7.1–7.2 was reached; these were $Mg_3(PO_4)_2.4H_2O$.

The causes of the slight fall in the P_H curve of the "peptone minus" cultures of *Aspergillus niger* during the first 3 days of incubation is problematic. Possibly during rapid formation of protoplasm of the young mycelial threads the NH_2 group of the

amphoteric amino acids is slightly more utilized than the non-nitrogenous part, leaving some carboxyl groups in excess. It appears probable that this differential utilization by the extremely fast-growing young germ tubes from the many spores of the inoculum produces a brief state of hyperacidity before autolysis, respiration, and other processes which make for alkalinity have come into full play. This temporary increase in acidity was not produced by *Sphaeropsis* and *Diplodia* in the P— medium; with these fungi the inoculum was a relatively large quantity of

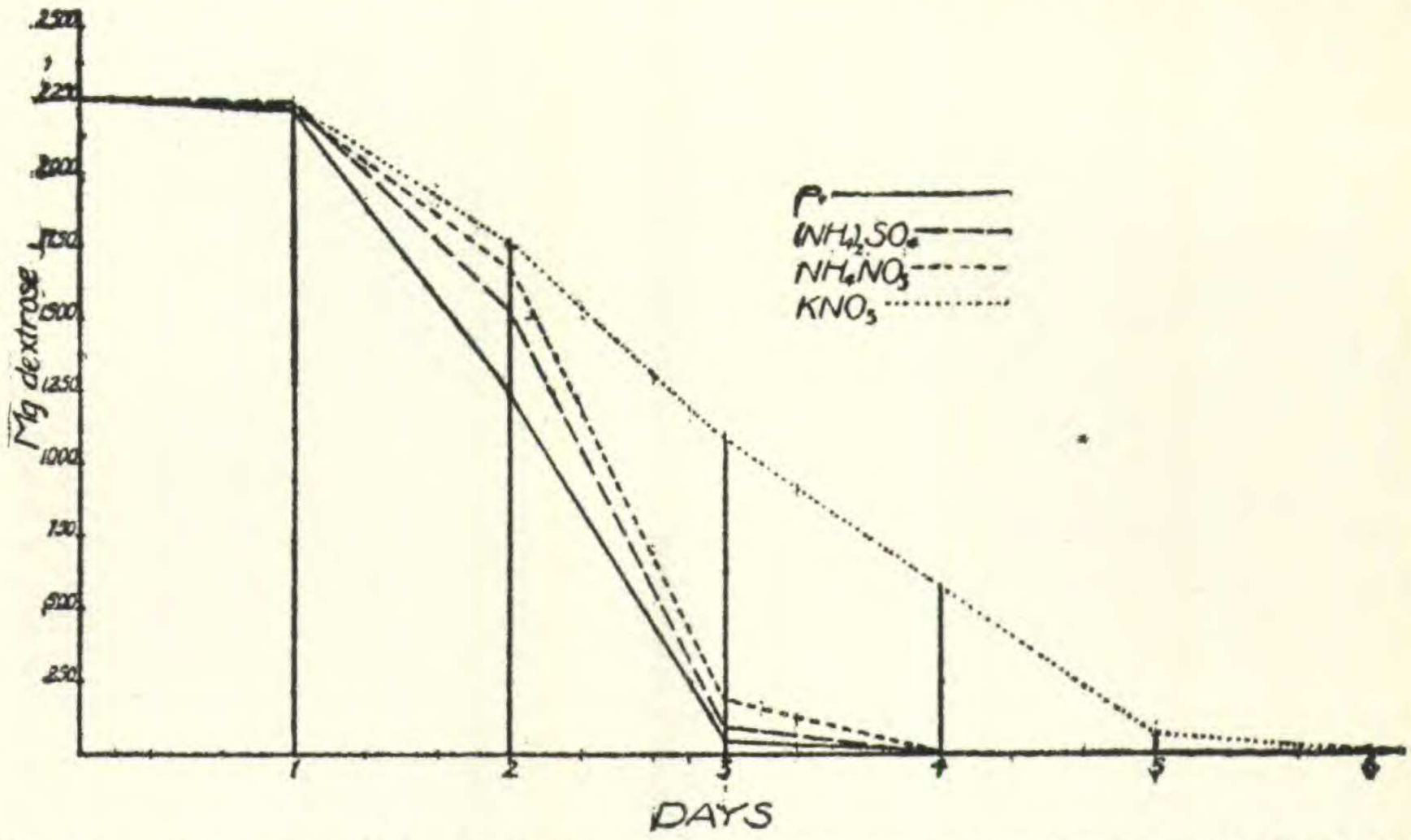


Fig. 9. Rates of carbohydrate consumption; amount of dextrose in 50 ml. media *Aspergillus niger*, second series.

mature mycelium in which respiration and autolytic processes were already predominant. The amount of autolysis, as indicated by decrease in dry weight from the maximum, is proportional to the weight the fungous mat attains.

The fact that after the period of rapid development of the fungus, the amount of NH_2.N remains small and relatively constant, while the "peptid" N of the P+ and P— media regularly decreases, indicates that amino N is a very readily assimilable form, and that the amino acids and possibly the reconstructed peptids, liberated during autolysis of the fungous proteins, are quickly assimilated by the living protoplasm because they are in directly utilizable forms. The results of Zaleski and Israilsky

('14) are very significant; they found that the best source of N for yeast is the autolysate of the yeast itself. Similarly Zaleski and Pjukow ('14) showed that fungous autolysate was superior to $(NH_4)_2SO_4$ as an N source for *Aspergillus niger*.

It seems well to reconsider here the opinions of Ehrlich, Czapek, Zaleski, and others on the form in which N is directly assimilated from various N sources. While there is no conclusive proof that N obtained by the fungi from amino acids, peptids, amines, alkaloids, etc. is actually in the form of ammonia, the nicety of

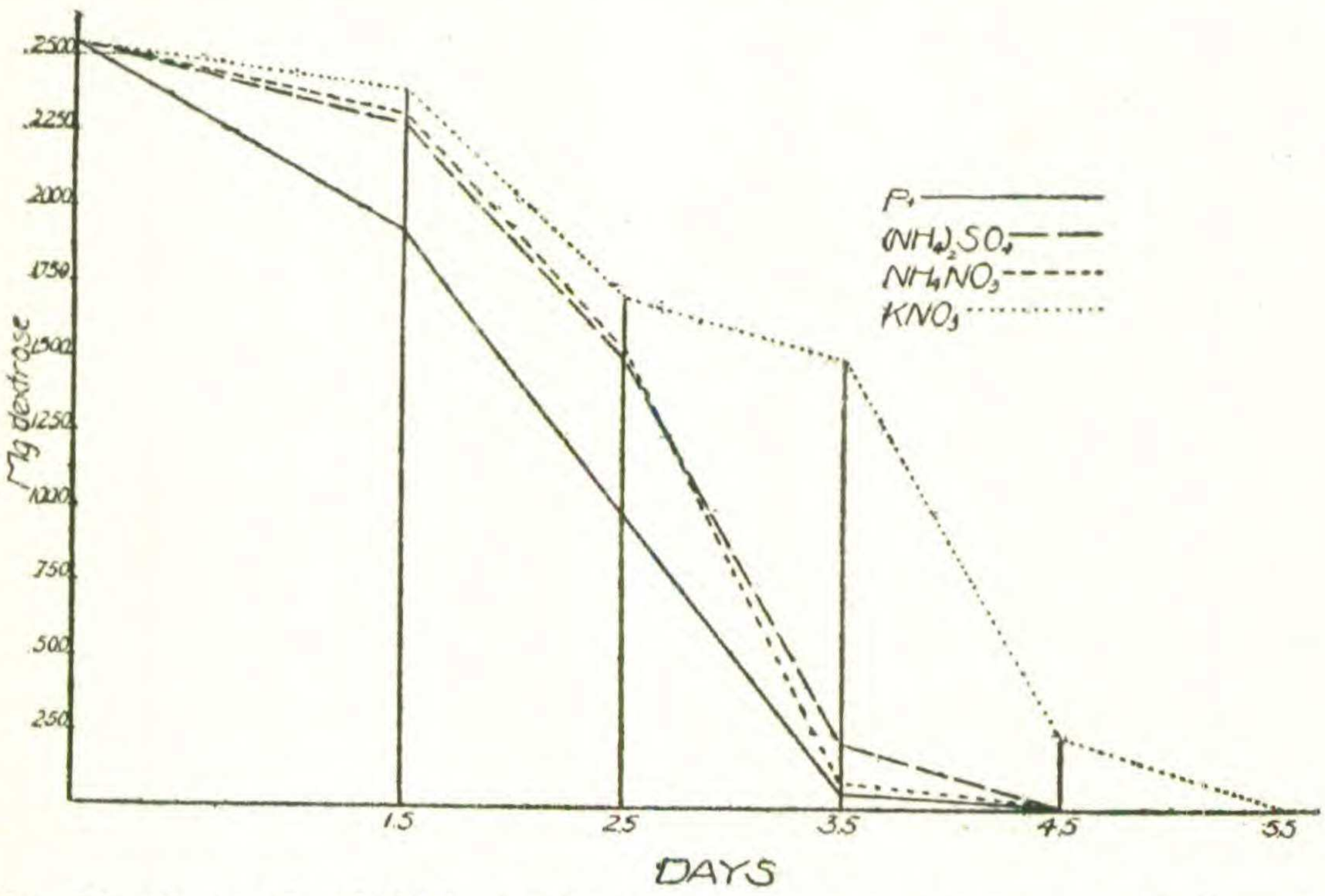


Fig. 10. Rates of carbohydrate consumption; amount of dextrose in 50 ml. media *Aspergillus niger*, third series.

the equations developed by Ehrlich and his associates, together with quantitative proof in some cases, makes it seem likely that the NH_3 and probably its simple substitution products are the forms directly assimilated. More evidence for this is the fact that Fischer obtained the diamino acids of β vinyl acrylic acid, sorbic and fumaric acids by the direct action of ammonia on these acids. It does not require a great stretch of the imagination to think of the highly reactive cleavage products of sugars behaving similarly toward NH_3. This assuredly does not preclude Czapek's theory of the direct utilization of amino acids and other

amido compounds, if such are actual units in the protein structure of the organism involved. In fact the conceptions of both factions should be combined to help account for the various degrees of utility of a particular nitrogenous compound for different fungi. Every genus of fungi and possibly every species of a genus is an entity in itself as regards its physiology. For example, *Aspergillus niger* makes a greater growth on $(NH_4)_2SO_4$

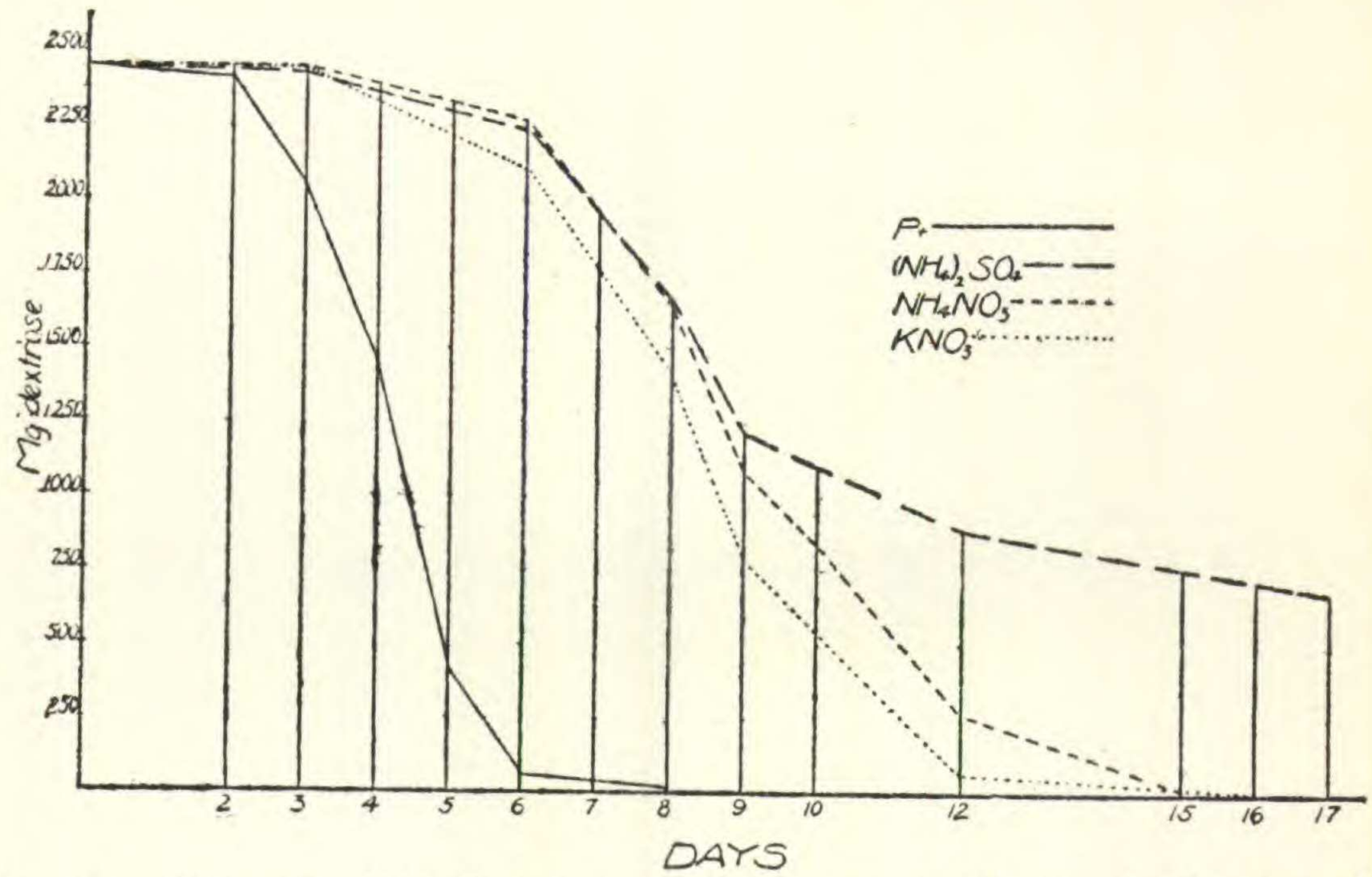


Fig. 11. Rates of carbohydrate consumption; amount of dextrose in 50 ml. media. *Sphaeropsis malorum*.

than does *A. glaucus*, while the latter is superior in the ability to assimilate nitrates. Nitrogen distribution studies on fungous mats to discover what amino acids are actually present in the substance of the fungus, and subsequent cultural studies in which different combinations and proportions of the units are used as the sources of N would probably contribute much toward a proper understanding of metabolism (van Slyke, '11). Such work requires much time and the cooperative labor of several associates, and could not for this reason be carried out during the time of this investigation in this laboratory.

A few determinations of the total N of dry fungous mats were made and these showed the following:

Organism	Medium	Days incubation	Per cent N
Aspergillus niger	P+	4	4.85
Sphaeropsis malorum	P+	35	3.45
	P—	35	4.16
	KNO_3	35	3.813
Diplodia natalensis	P+	8	5.65
	P+	12	5.08
	P+	18	4.48
	P—	8	5.28
	P—	12	5.32
	P—	18	5.08
	$(NH_4)_2SO_4$	8	7.11
	$(NH_4)_2SO_4$	12	6.50
	$(NH_4)_2SO_4$	18	6.70
	NH_4NO_3	8	5.54
	NH_4NO_3	12	5.57
	NH_4NO_3	18	6.64
	KNO_3	8	4.60
	KNO_3	12	4.59
	KNO_3	18	4.00

The N content of the fungi is seen to vary with the medium and the duration of incubation. The percentage N in *Diplodia*

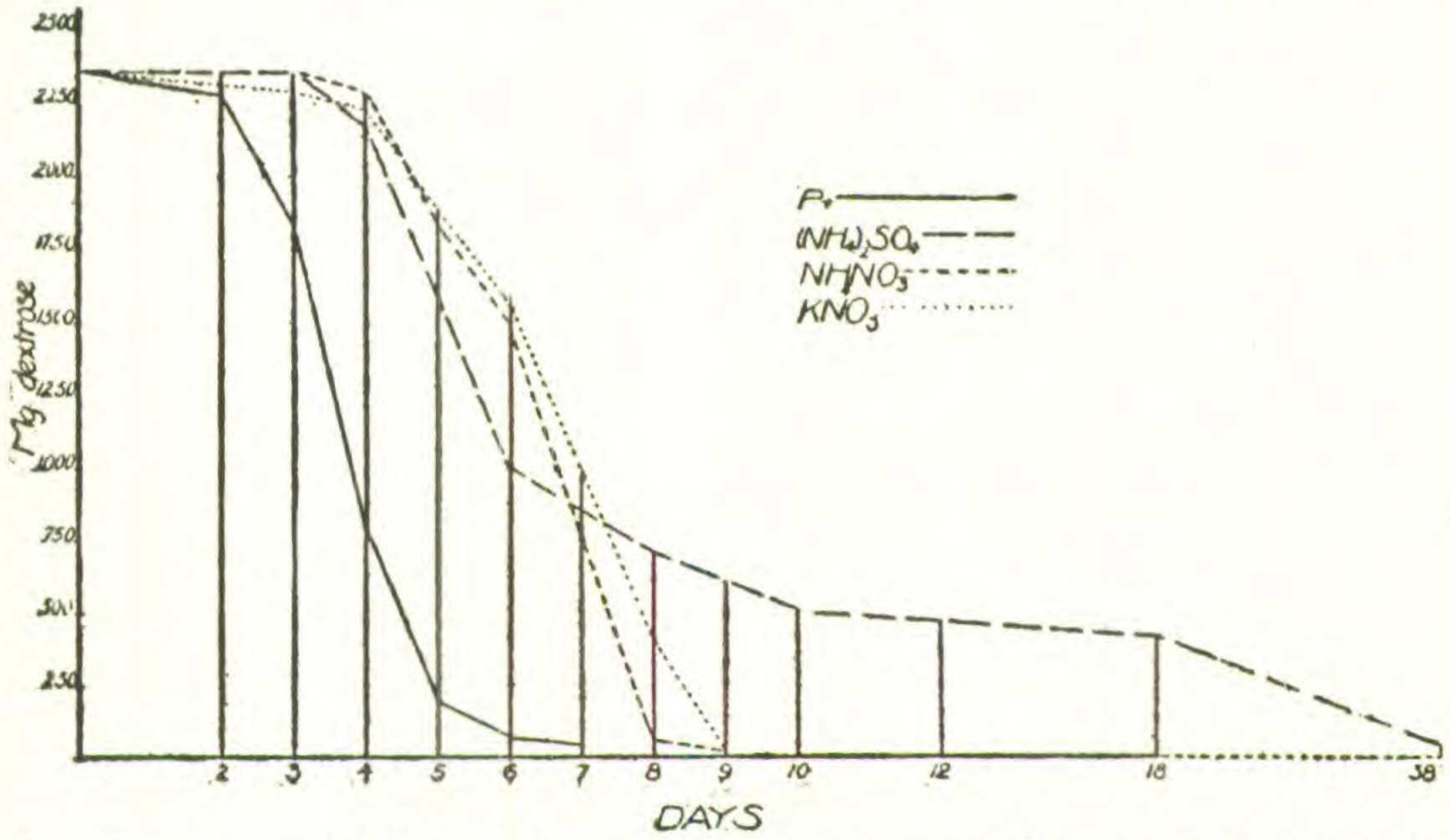


Fig. 12. Rates of carbohydrate consumption; amount of dextrose in 50 ml. media. *Diplodia natalensis.*

decreased with continued incubation on all the media except the NH_4NO_3 on which it showed an increase. This is similar to the findings of Terroine and his associates ('22) who worked with

Aspergillus niger. The media which were made strongly acid by the fungi produced mats having the highest N percentage. This is possibly due to the inhibiting action of the H ion on autolysis. The media on which the fungi made the fastest and largest growth yielded mats having the lowest per cent of N.

The behavior of the 3 fungi in the $(NH_4)_2SO_4$ medium was similar in that all increased the acidity of this solution. The greatest hydrion concentration was produced by *Aspergillus niger;* the maximum acidity (P_H 1.9) appeared in 3 days, after

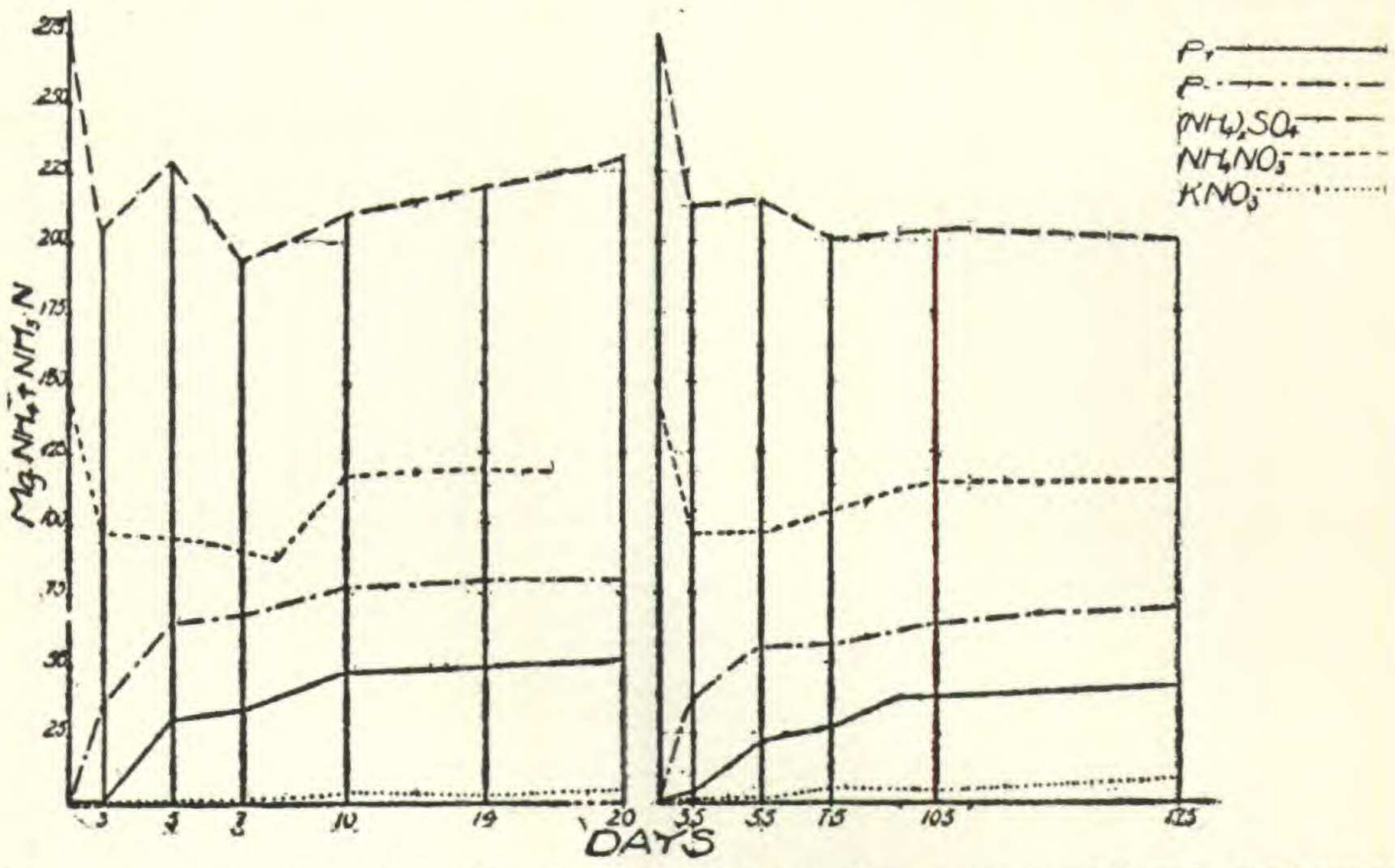


Fig. 13. Ammonium N in 50 ml. media. *Aspergillus niger,* second and third series.

which time there was a slight decrease in $[\overset{+}{H}]$ to a constant point, P_H 2.3–2.4. This decrease is synchronous with the disappearance of the sugar, the beginning of a decrease in the weight of the fungous mat, increase in the total and ammonium N from a minimum, and appearance of a trace of amino N in the medium. The maximum acidity reached is due to the sulphuric acid freed from the $(NH_3)_2H_2SO_4$ plus the organic acids formed in the decomposition of the sugar, the decrease of acidity to the consumption of the organic acids and also to autolytic processes. The iodine test for soluble starch gave a negative result after 4 days of incubation but a strongly positive test after 20 days. A similar course of reactions takes place in the cultures of *A. niger* on the

NH_4NO_3 medium. The acidity reached is P_H 1.6, greater even than in the $(NH_4)_2SO_4$ medium, indicating that the ammonia of the $NH_3.HNO_3$ is consumed more rapidly than the nitrate ion. The results for ammonium and nitrate N also show this. Boas ('18), in his criticism of Czapek's work, emphasized also the effect of reaction on the comparative assimilability of various nitrogenous compounds, but in his corrective experiments he ridiculously resorted to litmus roughly to indicate the reaction instead of determining active acidity.

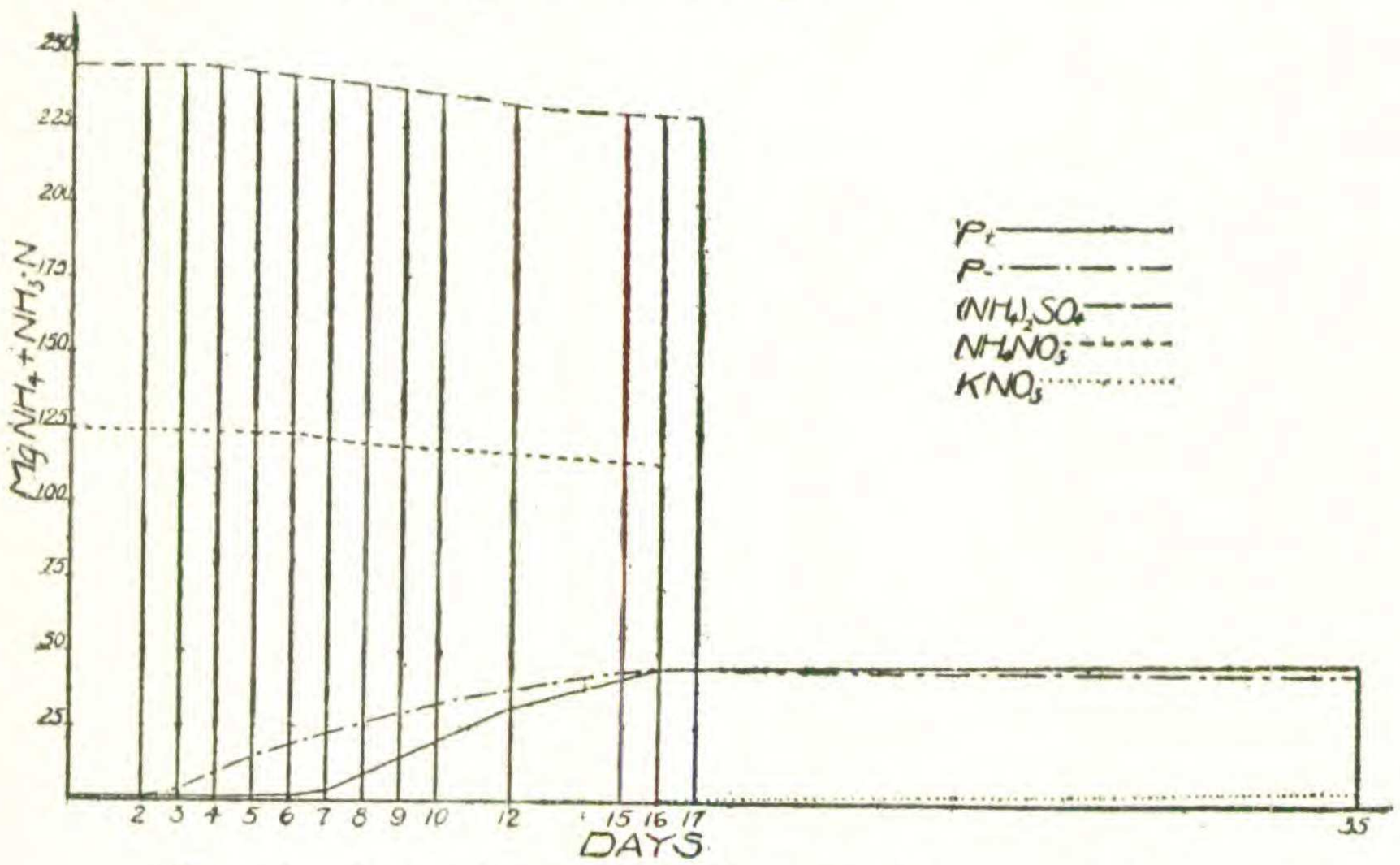


Fig. 14. Ammonium N in 50 ml. media. *Sphaeropsis malorum*.

The *Diplodia* and *Sphaeropsis* make a relatively slow growth on the $(NH_4)_2.SO_4$ medium and very gradually decrease the dextrose content of the solution, a small quantity of sugar being present even after 38 days of incubation. Similarly, the NH_4 and total N content and hydrion exponent gradually diminish throughout the entire period and the trace of $NH_2.N$ increases slightly. An appreciable odor of ethyl alcohol is evident after 7 days' growth. Autolysis in these cultures does not play an important role during the time of the experiment. These results corroborate those of Iwanoff ('21), who found that an acid reaction and the presence of alcohol arrested protein decomposition in fermenting fluids. That the courses of metabolism of these 2 fungi on the NH_4NO_3 medium are different is indicated by the

different nature of the change in hydrion concentration, the slower consumption of sugar by the *Sphaeropsis*, and the different rates of assimilation of the ammonium and nitrate ions. The *Diplodia* gradually increases the $[\overset{+}{H}]$ from P_H 4.0 to P_H 2.7 during the 38 days of incubation, while the *Sphaeropsis* during the first 6 days slightly increases the $[\overset{+}{H}]$ from P_H 4.0 to 3.8 and then during the succeeding 8 or 9 days decreases it from P_H 3.8 to P_H 5.8. The disappearance of glucose from the medium of the *Diplodia* cultures takes place about the ninth day, but not until

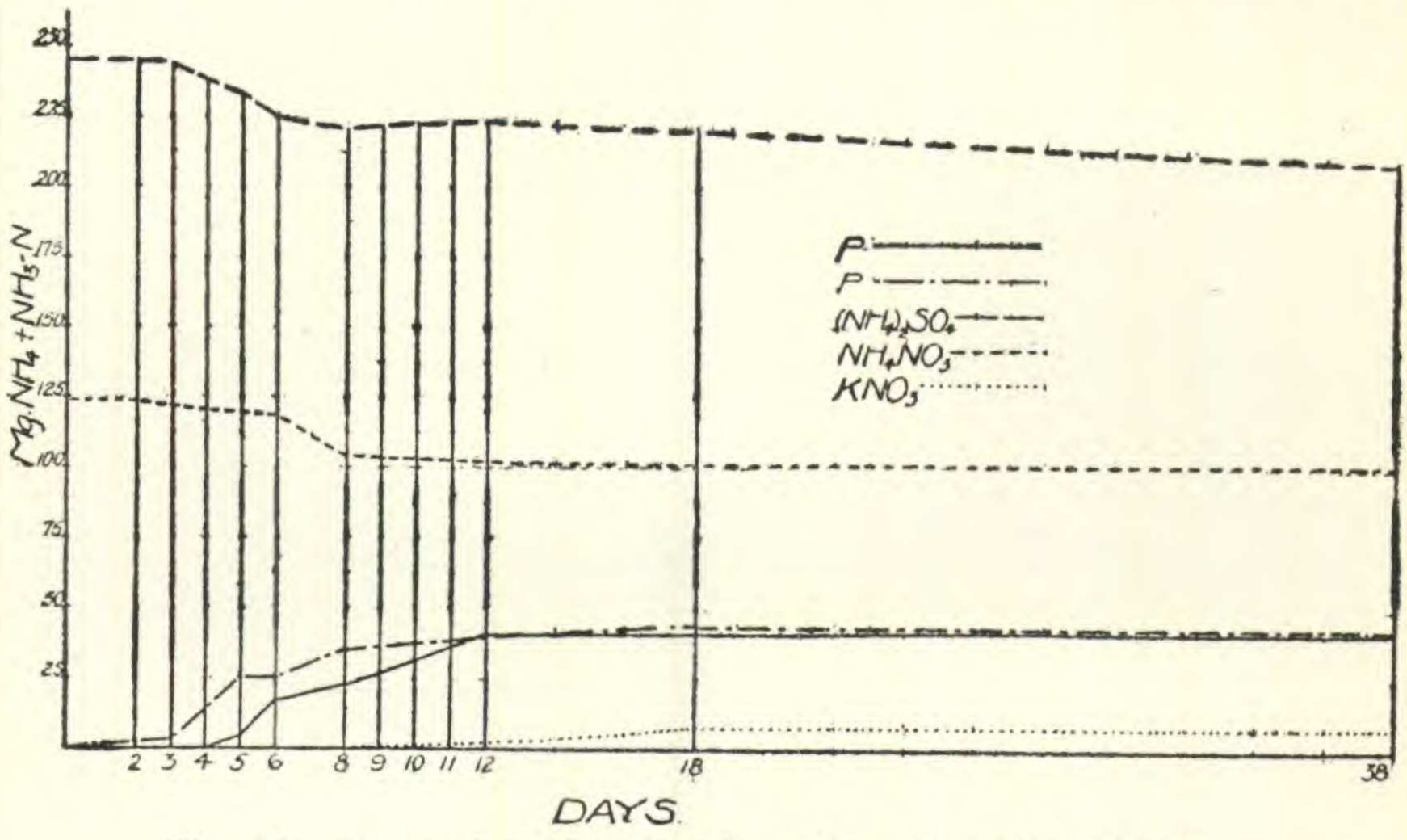


Fig. 15. Ammonium N in 50 ml. media. *Diplodia natalensis.*

the fifteenth day in the cultures of *Sphaeropsis;* and the appearance of autolytic predominance, as indicated by loss of weight, occupies the same relative position. *Diplodia* assimilates the NH_4 faster than it does the NO_3 of the NH_4NO_3 molecule, while the opposite is true for *Sphaeropsis.* This largely accounts for the different curves for active acidity. The different rate of consumption of dextrose and autolytic effects may also play some part in this change of $[\overset{+}{H}]$.

The curves for hydrion concentration of the culture solution of the second and third *Aspergillus* series on the KNO_3 medium are peculiar in that they show an increase of 0.6 P_H the first day, followed by a fall of 0.3 to 0.5 P_H during the next 4 days and then a gradual rise in P_H value to the end of the experiment. The

fall noted was not brought out by the first series with this fungus because determinations were not made until the third day of incubation. A possible explanation is similar to that offered for the action of the organism on the P— medium. The period of spore germination and extension of the young germ tubes is one in which the nitrogen of the KNO_3 is more rapidly used than is the dextrose decomposed to form organic acids, and consequently, due to the freed K, there is a slight increase of the hydroxyl ions above the original hydroxyl-ion content of the medium.

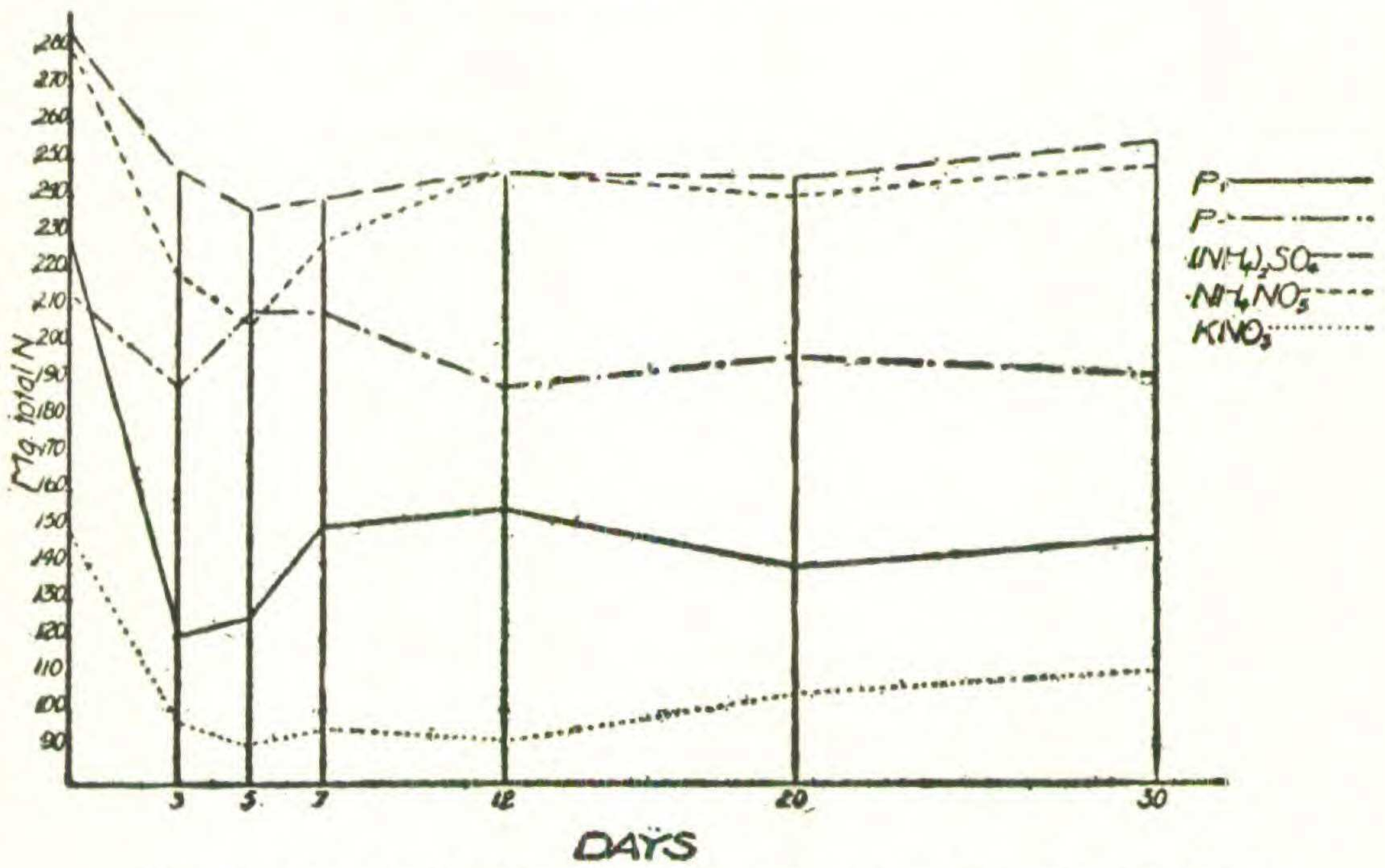


Fig. 16. Total N in 50 ml. media. *Aspergillus niger*, first series.

This is followed by the short period of the predominance of organic acids from the decomposed dextrose and the resulting increase in acidity. Then after the fifth day, the sugar and organic acids being nearly all assimilated, the increasing products of autolysis plus the OH resulting from the different rates of assimilation of the potassium and nitrate ions from the KNO_3, the K being in excess of the needs of the fungus, rapidly change the reaction toward the alkaline side. In the cultures of the slower-growing *Diplodia* and *Sphaeropsis* the above phenomenon is not in evidence. The medium was gradually diminished in acidity from P_H 4.0, becoming strongly alkaline (P_H 7.8 in the *Diplodia* cultures and P_H 8.1 in the *Sphaeropsis* cultures). Up to the twelfth day for

Sphaeropsis and the ninth day for *Diplodia* this is largely a matter of the differential absorption of the K and NO_3 ions. When the sugar has been consumed the dry weight of these organisms immediately begins to decrease, and autolytic products rapidly change the reaction to strongly alkaline (see page 317 of this article for Waksman's explanation). The total N (as applied to cultures of *Actinomyces*) of the culture medium decreases slowly but simultaneously with the rapid diminution of glucose, and this rapidly rises above the minimum when the sugar has dis-

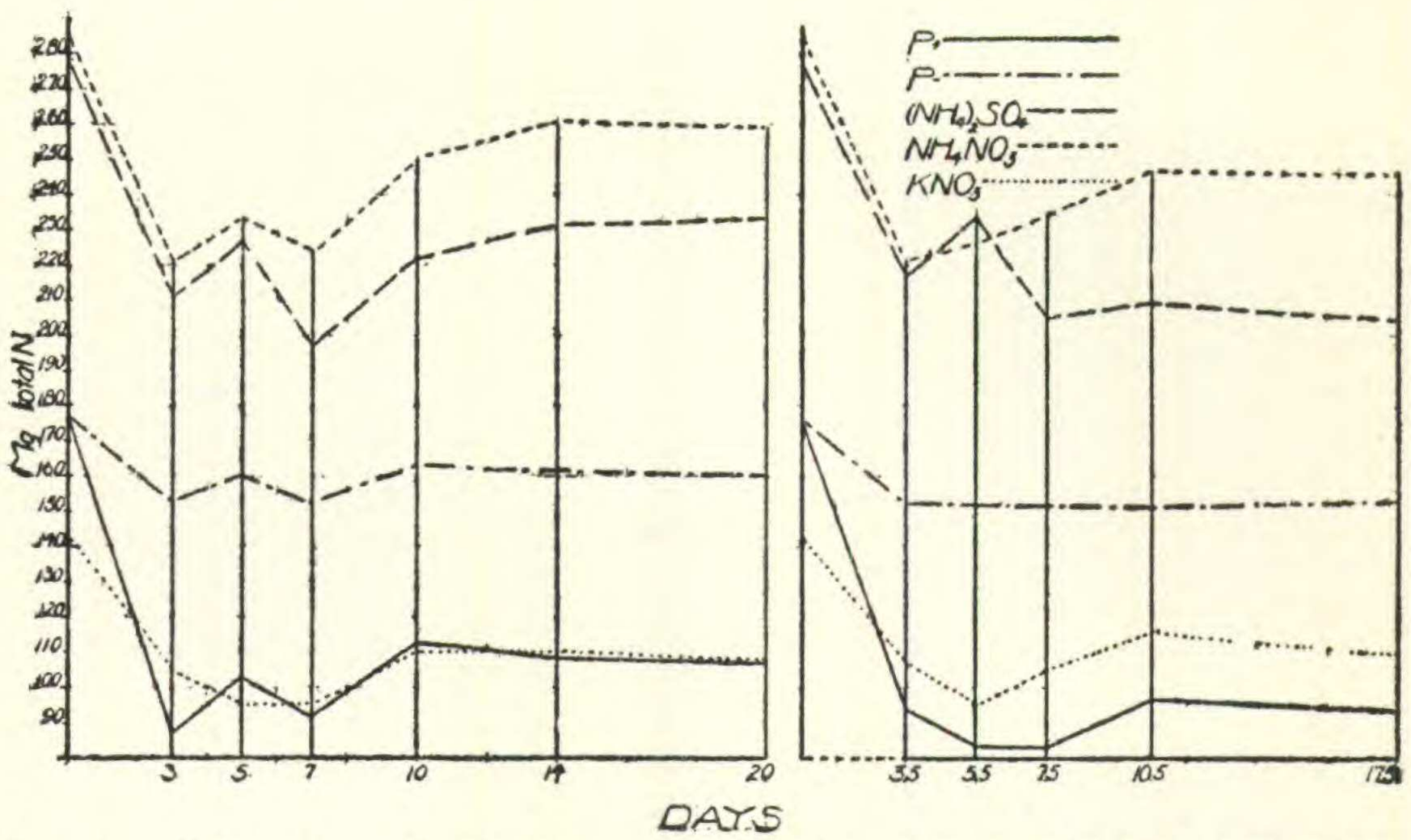


Fig. 17. Total N in 50 ml. media. *Aspergillus niger*, second and third series.

appeared. On continued incubation there may be a fall in the total and NH_4.N content due to the precipitation as $MgNH_4PO_4.6H_2O$ of NH_3 formed in autolysis; this is observable in the results for the *Diplodia* series.

The determinations show that in all the cultures of all 3 fungi on the inorganic nitrogenous media the amino N content of the solution did not attain more than a trace; 7.26 mgm. per 50 ml. of medium was the greatest value determined. This in itself indicates the ready assimilability of this form of nitrogen. Consider, for example, the 35-day cultures of *Sphaeropsis malorum* on the KNO_3 medium. Autolysis had decreased the weight of the fungus to almost one-half of the maximum weight attained in

10 days of incubation, yet the NH_2.N content of the culture fluid was only 0.14 mgm. per 50 ml.

To recapitulate, it is seen that the data throw some light on the forms of N which are directly utilizable and most serviceable for the fungi. Judging by the maximum dry weight attained, and disregarding the effects of acidity and other factors on metabolism, peptone in the presence of dextrose was the superior N source for all 3 fungi, whereas peptone in the absence of suga

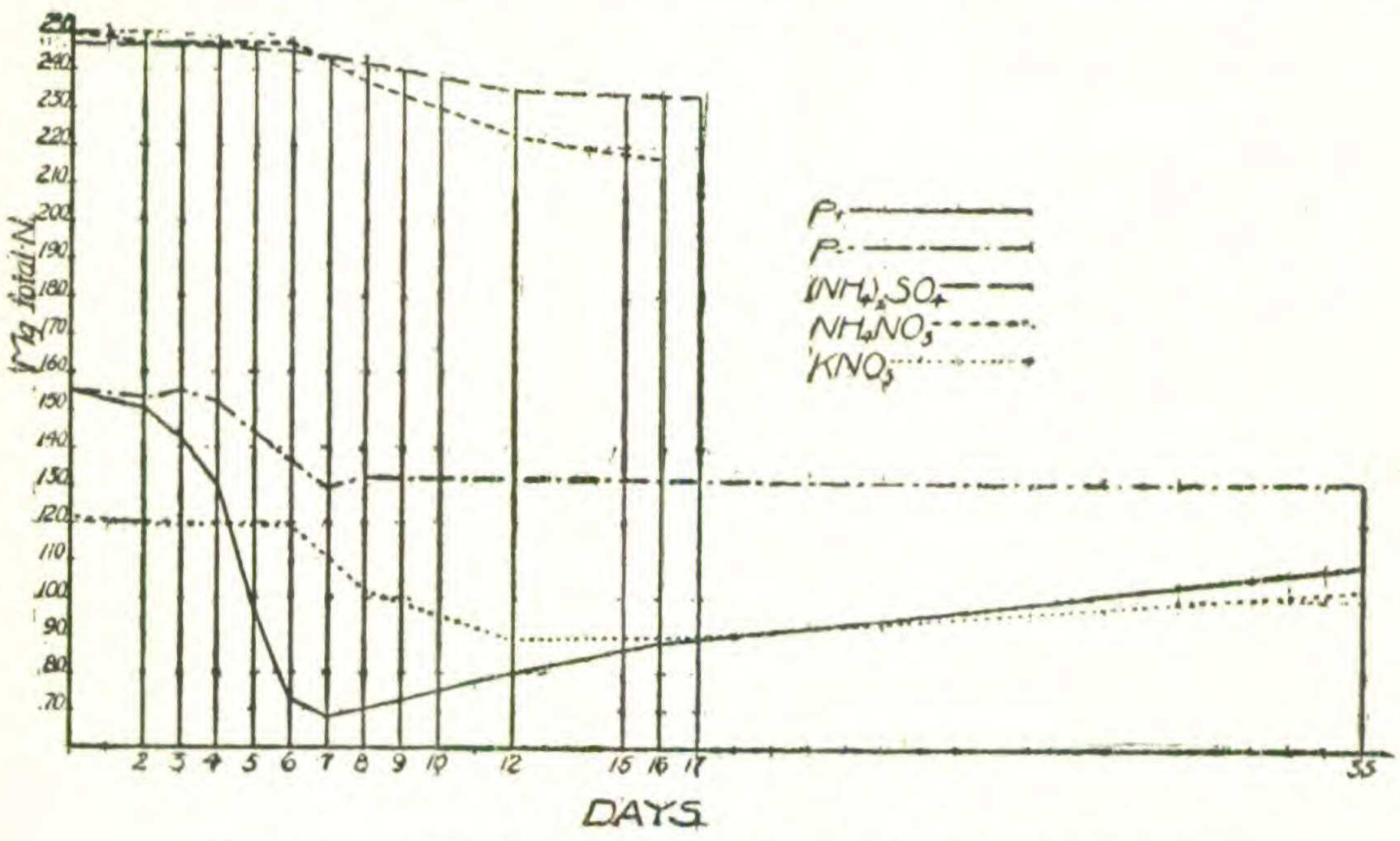


Fig. 18. Total N in 50 ml. media. *Sphaeropsis malorum.*

was distinctly inferior. This supports the point brought out on pp. 350–351 that amino acids and even "peptid" units may be directly assimilable if they are actually found as such in the proteins of the fungi. To this must be added that an available carbon source must be present to supply readily the energy necessary for the cementing of these building stones. It is evidently difficult to obtain this energy by deamidization of the peptone components and utilization of the non-nitrogenous complex. For the same reason amino acids serve but poorly as sources of both C and N, as has been shown by several investigators. However, it is probable that the NH_2 group of the amino acids is a very readily available form for those organisms that possess deamidizing enzymes sufficiently strong to split off this group readily. Waksman ('18) found indications of such en-

zymes in *Aspergillus niger* and other fungi. The theoretical equation for this would show the NH_2 group hydrolytically split off as ammonia, leaving the hydroxy group in the corresponding place in the acid molecule, as was pointed out by Ehrlich. Ammonia thus formed is not detectable; therefore, it seems reasonable to assume that it is not formed, and that the NH_2 group is united directly to the non-nitrogenous units, the excess hydrogen increasing the acidity of the solution. The initial rise in H-ion concentration of all the P+ cultures may be partly due to this as well as to organic acids from the sugar.

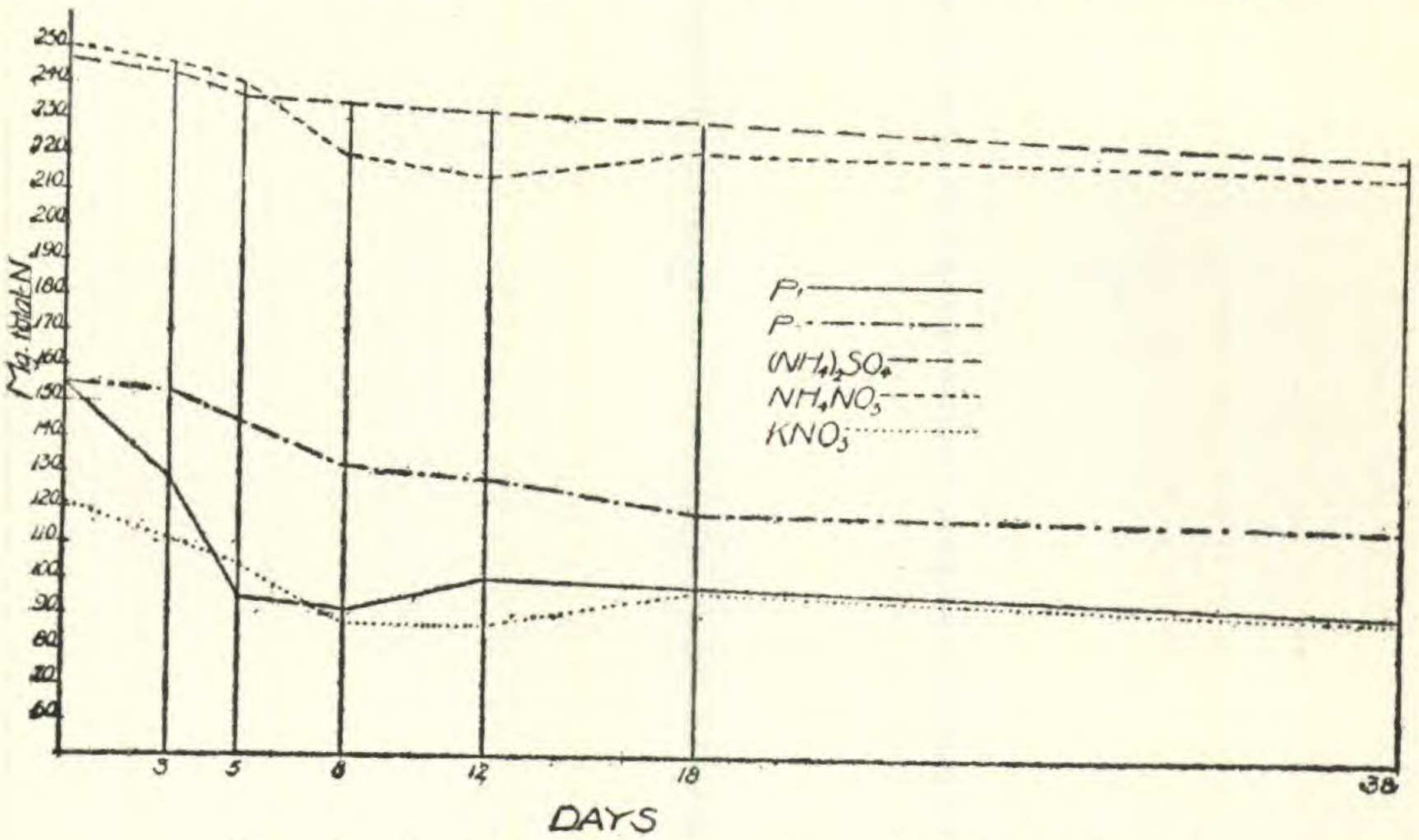


Fig. 19. Total N in 50 ml. media. *Diplodia natalensis.*

For *Aspergillus niger* the NH_4 ion is more serviceable than the NO_3, as the results show greater absorption of the former than the latter from NH_4NO_3. And judged by the weight of the fungus the $(NH_4)_2SO_4$ is superior to KNO_3 in spite of the acidity produced as a result of the use of the first named. *Sphaeropsis malorum* in the NH_4NO_3 medium absorbs the NO_3 ion at a slightly greater rate than the NH_4, and the organism on the KNO_3 medium makes a faster and larger growth than on the $(NH_4)_2SO_4$, showing that it is more sensitive to the free H_2SO_4 than is the *Aspergillus*. Judged by the rate of absorption of the ions of the NH_4NO_3 medium, the *Diplodia*, on the other hand, shows a slight preference for NH_4. The sensitiveness of this organism to the hydrion,

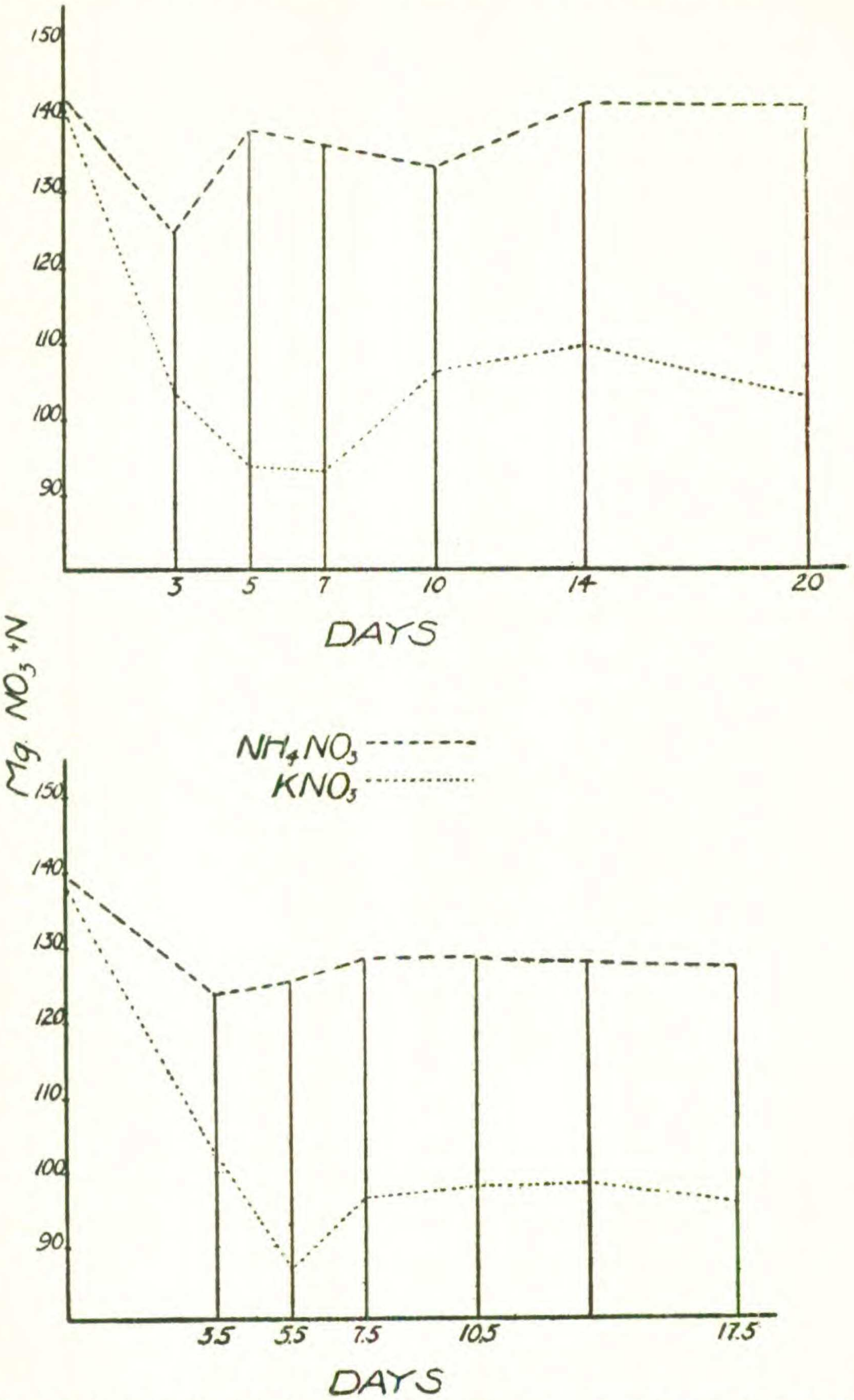


Fig. 20. Nitrate N in 50 ml. media. *Aspergillus niger*, second series (top; by difference); and third series (bottom; Strowd's method used).

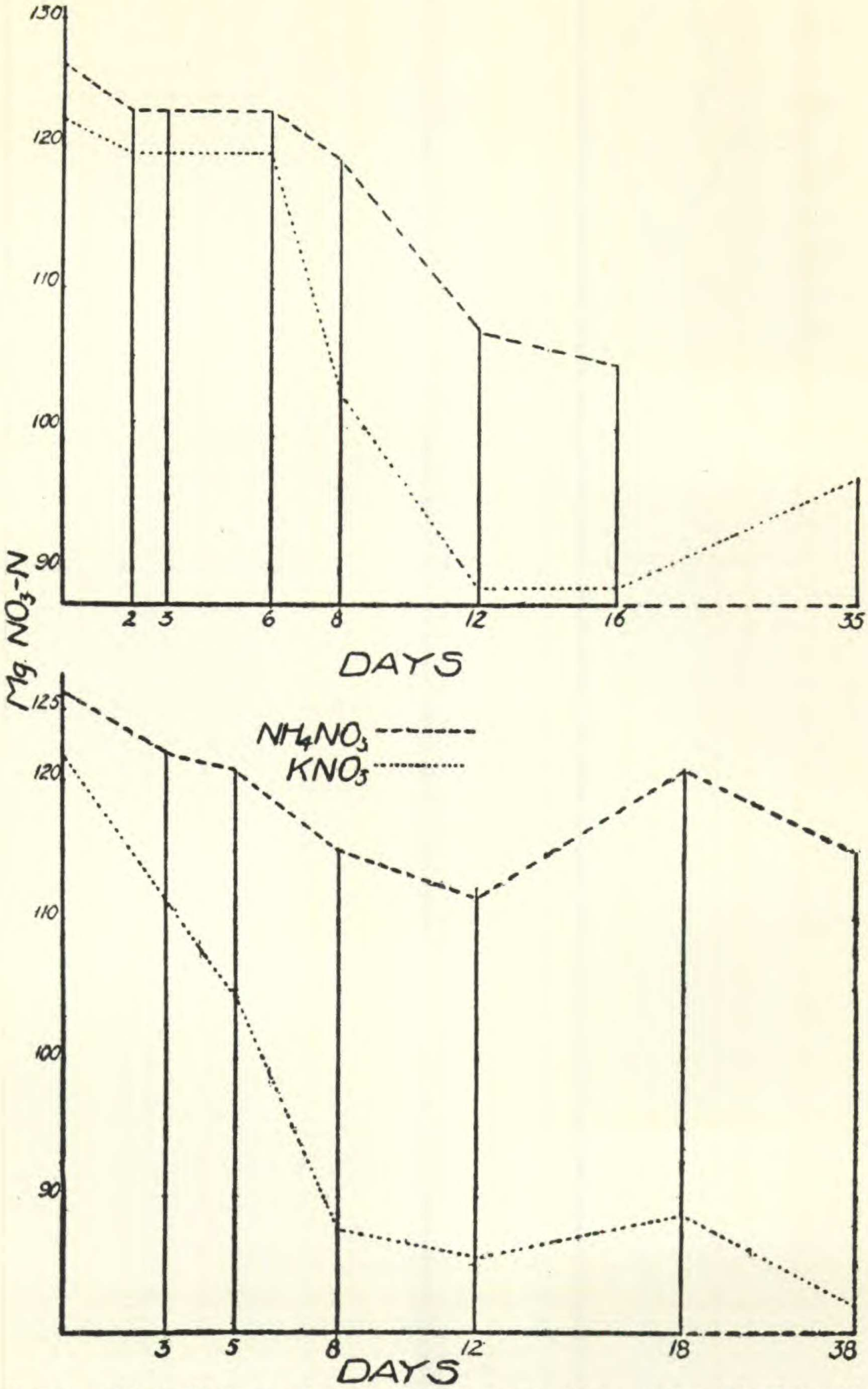


Fig. 21. Nitrate N in 50 ml. media. *Sphaeropsis malorum* (top; NO_3.N by difference); *Diplodia natalensis* (bottom; NO_3.N by difference).

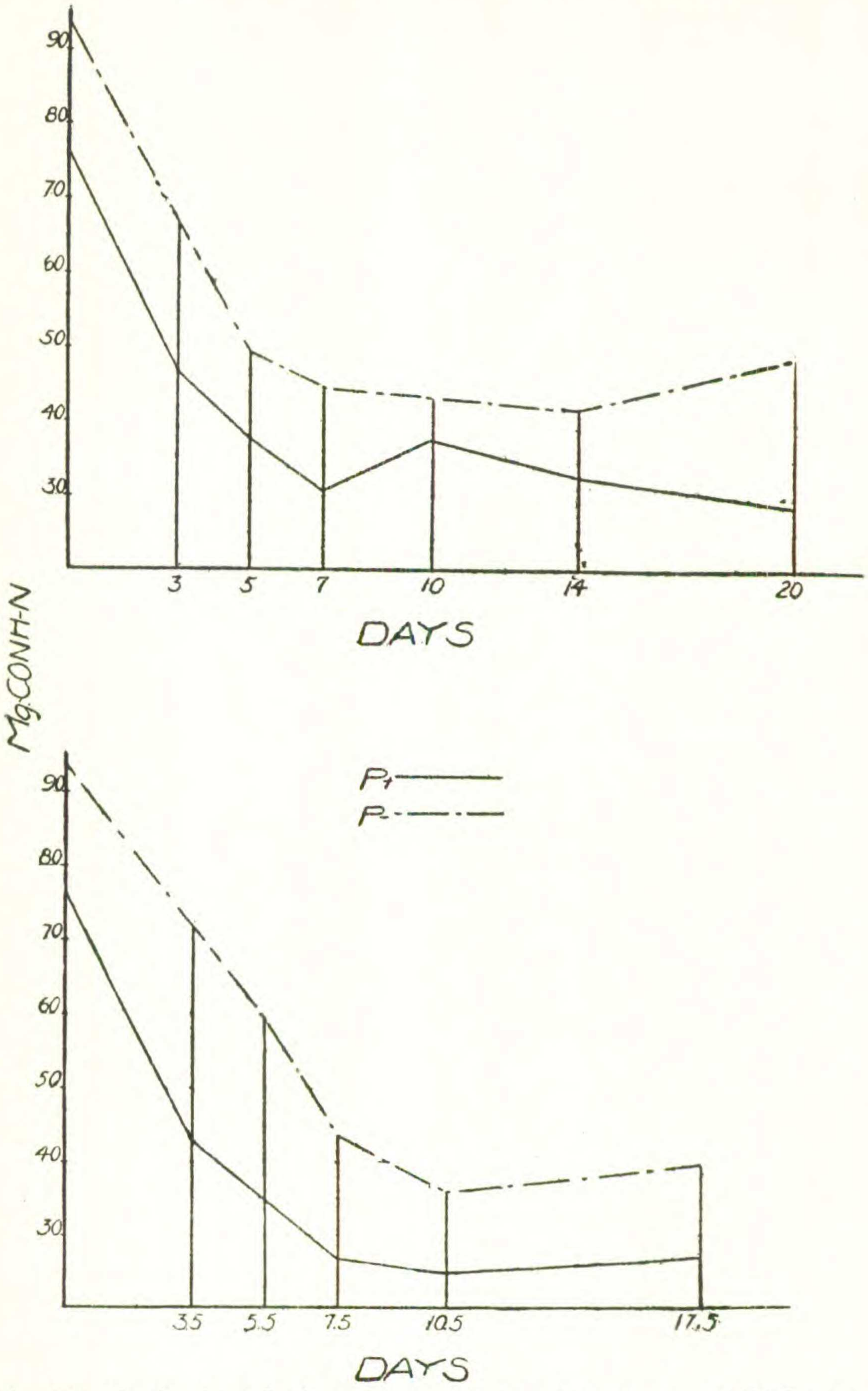


Fig. 22. Peptid N in 50 ml. media. *Aspergillus niger*, second and third series.

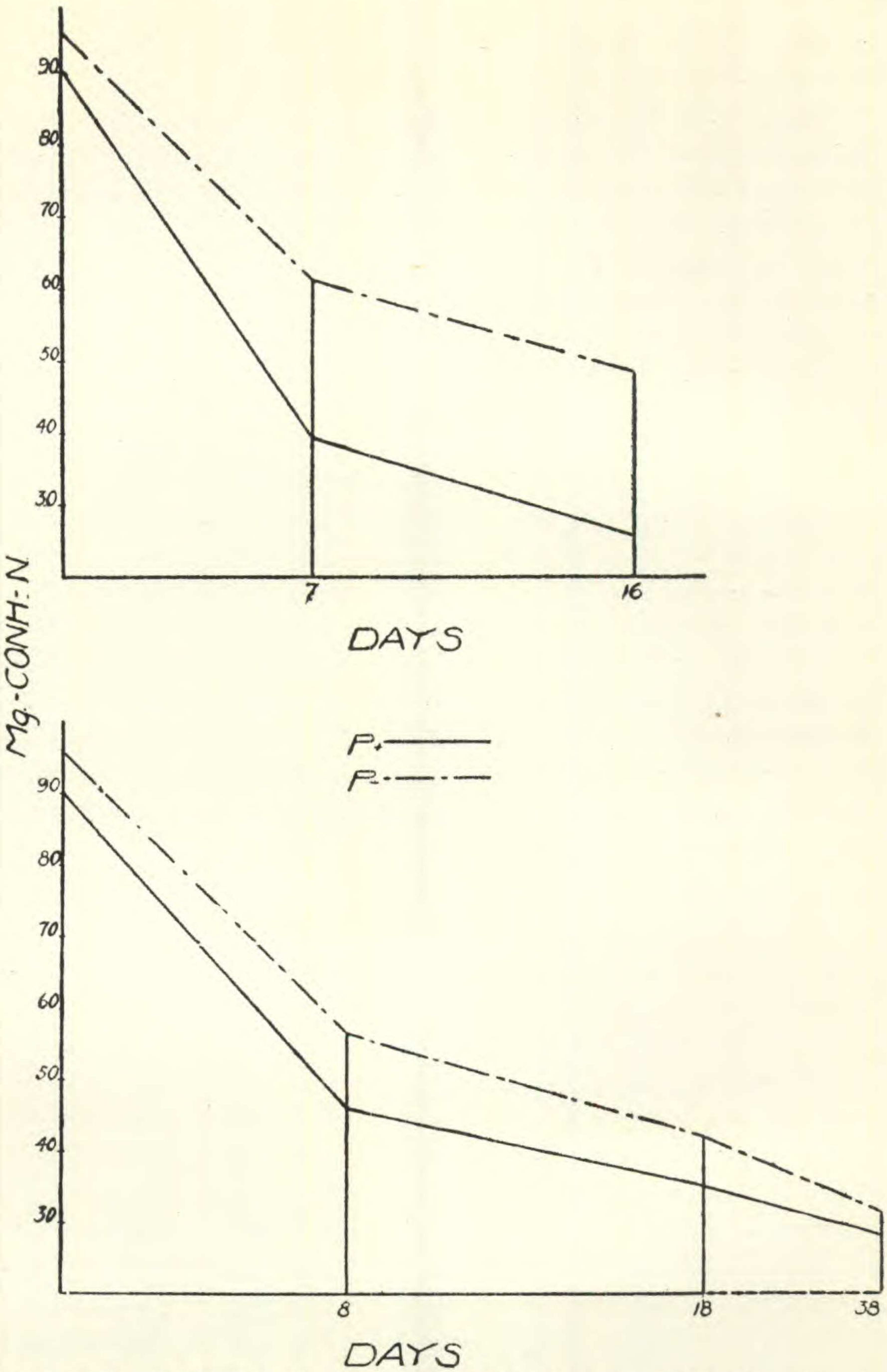


Fig. 23. Peptid N in 50 ml. media. *Sphaeropsis malorum* (top); *Diplodia natalensis* (bottom).

as shown by its growth on $(NH_4)_2SO_4$, is similar to that of the *Sphaeropsis*.

The general conclusion from these considerations is that there is a distinctive physiology for each of these 3 organisms and that this conditions the form of the inorganic nitrogen which is most assimilable. There are no conclusive data in the literature to show that nitrates must be reduced to nitrites or ammonia before assimilation. Reasoning *a priori*, the form of nitrogen supplied must be reduced (if an oxide) or oxidized (if NH_4 or NH_3) to form the amino group, and other conditions (as H-ion concentration) being the same, organisms show a differential use of these ions because of their different powers of reduction, oxidation, and synthesis. As has been already pointed out, many erroneous assumptions have been made in this regard because investigators failed to consider the NH_3 produced proteolytically. For this reason also, the necessity for making dry-weight determinations at frequent intervals is evident.

The order of relative assimilability of nitrogenous compounds for a specific fungus at one H-ion concentration may be entirely different from the order at another P_H value. Similarly the effects of temperature, humidity, light, aeration, agitation, and possibly other factors on assimilation must be so considered; the problems are complex.

SUMMARY

1. A review of the literature on the N metabolism of fungi is given.

2. The methods used are described and reasons are given for the selections made.

3. Autolysis is at first indicated by decrease in dry weight of the fungous mat from a maximum and by the formation of ammonia in the peptone and KNO_3 media; somewhat later, by increase in total N of the culture solution from a minimum in all the media, and by the appearance of a trace of amino N in the 3 inorganic nitrogenous media.

4. Autolysis in a species is proportional to the rate and amount of growth attained.

5. Ammonia is the chief nitrogenous product of autolysis and is a waste product of the splitting of the peptone of the media in the absence of another C source. In the presence of dextrose NH_3 was reassimilated. Disappearance of carbohydrate from the culture medium is synchronous with the beginning of autolysis.

6. In cultures whose H-ion exponent becomes greater than P_H 7.0 the loss of N is due to the evolution of NH_3.

7. Conditions necessary to the formation of crystals of $MgNH_4PO_4.6H_2O$ and $Mg_3(PO_4)_2.4H_2O$ in the cultures were determined.

8. The probable causes for the shifting of the H-ion concentration of the media are discussed.

9. Nitrogen of the amino group is readily assimilated by the fungi studied.

10. Some factors influencing the N content of the fungous mat are the N and C sources of the medium, the length of incubation, rate of growth, and hydrion concentration.

11. The organisms displayed markedly different physiological relations; this was indicated by their rates of growth and of sugar consumption, by their utilization and excretion of the several forms of nitrogen, and by the varying nature and extent of H-ion change of the medium.

The writer here expresses his appreciation to Doctor B. M. Duggar for criticisms and suggestions given throughout the work, and to Doctor George T. Moore for the privileges and facilities of the Missouri Botanical Garden.

Graduate Laboratory, Missouri Botanical Garden.

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